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Creationists and science in schools

Creation science has rightly been denied a licence in Arkansas on the grounds that it is religion in disguise. The educational implications are even more sinister.

The creationists may have lost the battle in Arkansas, but they are unlikely now to abandon the war on which they are engaged. Science educators everywhere, but especially in the South, had better reconcile themselves to that. The United States courts are notoriously a means by which determined if misguided people can refuse to accept the inevitable, creating confusion and widespread nuisance. In the circumstances, it is important that everybody should be clear what the war is about. It is not a war about the validity of Darwinism, however understood and glossed. Nor is it a war between religion and science, for some of the most influential evidence against the creationists in Judge William Overton's court came from religious people anxious to dissociate themselves from the claims of the creationists. The underlying dispute is between a small section of the religious community in the United States to whom the evolutionary view of the world, inanimate and living, is anathema. The claim that "creation science" should be taught alongside orthodox evolution as if, too, were science is both preposterous and disingenuous.

The richest irony in the creationists' case is the attention that it pays to the supposed flaw in Darwinism, which is alleged to be unfalsifiable experimentally. By what logic can it be held that because Darwinism is unfalsifiable in some technical (and disputed) sense, a set of hypotheses which is in the same sense falsifiable and amply falsified should be given equal time in the curriculum of the public schools of Arkansas and elsewhere? How, in the side-by-side science lessons for which they have been asking, would the creationists deal with questions such as the presence in the Earth's crust of lead isotopes which have apparently accumulated over the past 4,500 million years? By asserting that the technically falsifiable but amply verified explanation of radioactive decay is also false? And in that case, why should the proportions of radiogenic and nonradiogenic lead have been created at some later time in such a way as to mimic the effects of geochemical differentiation? To fool us? Or to show that the Creator was a good geophysicist? Most religious people rightly and consistently dismiss such questions as irrelevant, arid and even sacrilegious.

Other educational questions remain. Although the chief basis of Judge Overton's decision was that the state law he struck down was unconstitutional by providing a disguise for religious teaching, in many ways its requirements of science teachers were a more sinister threat to good sense. For teachers who are not allowed to tell what they consider to be the truth and who are required to tell lies are necessarily bad teachers. How are these epitomes of duplicity to be trained? Or is it thought, in Arkansas and elsewhere, that the teaching of science consists of the recitation of facts by teachers and feats of memory by students? Legally, what Arkansas had planned to ask of its teachers was an offence against the First Amendment. The damage that Arkansas had unthinkingly proposed inflicting on the education of its young people would have cast a longer shadow. When the issue of creationism in the schools comes up next, in Louisiana or wherever, the educational folly of giving equal time and place to a literal interpretation of *Genesis* should be given as much attention as its constitutional impropriety.

At the same time, some thought should be given to the way in which an over-literal interpretation of the constitutional interdiction against religious teaching in the public schools is itself

an impoverishment of American education. *Genesis* may be a pack of lies, or of allegories, but it is also an important part of the cultural heritage. So, too, is the Koran. Is it necessary that these important topics should be forever ignored, or relegated to "Eng. Lit." or fuzzy courses such as "the world about us" simply because school boards fear they may be accused of propagating religious views? For that matter, should the history of the world outside the Americas continue to be considered suitable only for colleges and universities? It is unlikely that the creationists now pushing for creation science would be mollified by concessions along these lines. But school boards throughout the United States should not think that Judge Overton's judgement is an all-round endorsement of the present curriculum.

Selling off telephones

Settlement of the anti-trust suit against AT&T will change the company — and the world

The Justice Department in the United States has followed the only sensible course by agreeing to stop its long-standing action against the world's largest company, American Telephone & Telegraph Company (AT&T). The basis of the complaint, that the dominant telephone company in the United States has used its profits from one kind of service (long-distance telephone calls for example) to subsidize other services (local telephone services) has in any case been overtaken by events. In a long succession of decisions in the past decade, the Federal Communications Commission has balanced a relaxation of the rules restricting regulated telephone companies (such as AT&T) from entering unregulated business (such as data transmission) with increasingly explicit rules against cross-subsidies. If the judgement in the suit had gone against the company, it would have seemed a nonsense in present circumstances — and would have left unresolved the question of what, in any case, should happen to AT&T. Only the lawyers would have profited.

So does last week's settlement presage the most radical transformation of American industry since Standard Oil of New Jersey was broken up in 1911? Not necessarily. AT&T has undertaken to sell off its interests in those local and regional telephone companies across the United States which at present deal directly with local customers. It will retain its long-distance network and will also win the right to compete directly with other companies in the telecommunications business, both domestically and overseas. Perhaps more important in the long run, the company will retain control of the Bell Laboratories (and of its manufacturing subsidiary, Western Electric). This formidable technological enterprise has hitherto functioned partly with one hand tied behind its back. Plainly the company is now gambling on the chance that even though its assets will be reduced by nearly two-thirds, it will be able to grow more quickly when disencumbered of the unprofitable telephone companies. Three uncertainties lie ahead. The process of divestment may be more difficult than foreseen. Congress and the courts may decide that they do not like the settlement, or some parts of it. And the fields that AT&T hopes to enter are already partly occupied by other unregulated telecommunications companies. (There would



have been howls of protest if the anti-trust suit against IBM had not also been dropped last week.) So for AT&T the future has the air of being an unaccustomed gamble, but a good one.

The chances are that it will succeed, and that the consequences will be profound, not merely in the United States. First, the emerging public telephone system in the United States will be an invaluable economic model for suggesting how such enterprises can be run on commercial lines. Nowhere else is such a task attempted. United States users of the telephone will most immediately discover that it costs them more to use the local telephone service as if it were unmetered water. Further ahead, however, they will find their habits changing, and telephones will be used increasingly for what they are best suited for in present circumstances — long-distance communications. It will be interesting to see how long telecommunications authorities elsewhere will be able to pretend this model is irrelevant to them.

The liberation of AT&T from the regulatory framework will also help to change the character of telecommunications. For several years it has been clear that technical innovation has outstripped the capital resources of the industry (and the capacity of potential consumers to buy its products). The result is that people who read in the technical literature of novel kinds of office switchboards, facsimile transmission by satellite, electronic mail of various kinds and high-density communications channels, have to put up with the much more rudimentary means of communication already in service. Competition from AT&T should help to bring down the prices at which these novel services are sold. More important, the entry of even a truncated AT&T into such fields should help to speed the exploitation of existing innovations as well as to sustain the pace of discovery.

But why all the bother? Can it really be of such great importance that people who have not yet learned to use pocket-size computers should be more able to install computer terminals in their living rooms? Or that people in Europe should be able to watch American television (and vice versa)? There is a widespread and unhealthy scepticism prompting these and other questions. The common underlying fallacy is to ignore the present importance of the telecommunications industry and to dismiss as unimportant the potential growth. Yet if the industrialized world wishes seriously to find something with which to occupy itself in the decades ahead, can it afford this insouciance?

How to save a university

The University of London is in danger of collapsing. It needs a recipe for survival.

For a quarter of a century, and in spite of a succession of supposed reforms, it has been clear that the University of London could not continue as it had become. The question now is whether it can survive. The request by University College, the university's largest college, to be supported directly by the University Grants Committee and not through the university's own bureaucracy (see page 88) is not intended as a threat to the continued existence of the federal structure. But that is what the effect will be. If University College gets its way, others — the London School of Economics, for example — will want the same, and will not easily be denied. And even though separate financing does not of itself mean separation, what would be left of the university's central administration would be even less able than at present to give the university a sense of coherence.

How, it will be said elsewhere, have the mighty fallen. Within living memory, the University of London was not merely the largest single university in the United Kingdom and the largest source of qualified physicians (which it still is) but a godfather to emerging universities in Britain and elsewhere in the British Commonwealth. The university was then an important source of academic qualifications (by means of its external degrees) for those studying on their own while, almost as a sideshow, it administered school-level examinations in most Commonwealth countries, old and new. In its heyday, the university was

imaginative and flexible, setting up a number of internationally important postgraduate institutions. Although always a federation in the sense of being the umbrella beneath which several constitutionally autonomous educational establishments agreed to function, the university appeared as a unified academic institution because its examinations and degree systems were administered centrally. Under the pressure of disparate events, most of these activities have been shed. The emergent universities have emerged, and no longer need an avuncular guiding hand. Countries once eager for London's services as a school-level examiner increasingly prefer to look after themselves. The demand for external degree examinations fell away as access to universities became easier in the 1960s. And, for more than a decade now, the university had delegated to its constituent parts much of the responsibility for setting their own degree examinations, largely in recognition that any common system must inhibit the pursuit of excellence by the stronger colleges. At the centre of the university is now to be found little but an office.

The past year has shown how vulnerable is an entity like this. The federation has become so loose that its vice-chancellor at the time (Lord Annan) was less able than his colleagues elsewhere to protest convincingly at the British government's decree that overseas students' fees must be increased (in 1979) and at the proposed cut in university budgets. Lord Annan did his eloquent best, but since last summer his successor, Professor Randolph Quirk, has had little choice but to hope against hope that the plans for retrenchment at individual colleges will somehow add up to compliance with the instructions of the grants committee for the university as a whole. (The obviously necessary survey of the scale of teaching in various subject areas, put in hand last September, will nevertheless probably come too late to provide individual colleges with timely guidance.) The university has promised to put redundant academics on a central register, and has asked colleges about to hire staff to think first of people being put out of work elsewhere within the university. So far, however, there is little prospect that the university will be able to put together an academic plan for the years ahead. The committee set up to do just that, under Sir Peter Swinnerton-Dyer, has been pre-empted by events. And if a plan should emerge by accident from the work of the subject review committees, the chances are meagre that this would be adopted by the university's senate.

It will, however, be tragic if the university simply withers away because it has no way of deciding what to do or how to do it. But time is so short that almost any plan will be better than none. So it is that it falls to the vice-chancellor to do off his own bat what the university's maze of committees cannot accomplish, and to say what the future will be like. A few simplifying assumptions will help. First, University College (bigger than many other British universities) is, like Imperial College, big enough to look after itself but should pay for its financial independence with unbreakable promises of help for the smaller fry. It is absurd, for example, that research facilities remain as jealously protected as they are, or that colleges wishing to provide specialized teaching must usually provide it for themselves. Second, the London School of Economics merits a similar degree of independence by its success in recruiting students from overseas.

Third, the vice-chancellor's chief concern should be with the rest of the university — seven or eight smaller colleges and a host of postgraduate institutes. The past few months have shown clearly that even willing partners in schemes for collaboration are given pause by the stark truth that collaboration cannot preserve jobs at all the institutions concerned, and may also hurt the academic pride of many of the teaching departments. The moral is that there should be an academic plan for these smaller components of the University of London that will, in due course, make collaboration between them natural and relatively painless. Any plan would enable these small but distinctive parts of the university to know where they are likely to be standing some years from now. No plan is a recipe for the further erosion of the university — or even for an agreement among the rump that it too would like collective independence. In that case, there would be nothing left.

Arkansas verdict against creation science

But battle now to move to Louisiana

Washington

Last week's ruling by a federal judge in Little Rock, Arkansas, that a state law requiring equal treatment of "creation science" and Darwinian evolution in public schools is unconstitutional, represented a significant victory for those who have argued that, even without any explicit reference to the Bible, such laws violate the required separation of church and state.

Yet if this particular battle has been won, the war against the creationists is far from over. Indeed both the publicity given to last month's trial in Arkansas and the apparent reasonableness of the demand for "balanced treatment" have in some ways helped the creationists' case. Only minutes after Judge William Overton had announced his verdict, the state Senate in Mississippi passed by a substantial majority a virtually identical bill to the one which had just been declared unconstitutional in Arkansas.

Judge Overton was uncompromising in his criticisms of Act 590, passed hurriedly by the state legislature in March during the closing hours of its 1981 session. Declaring creation science as having "no scientific merit or educational value", he said that "the conclusion is inescapable that the only real effect of Act 590 is the advancement of religion".

The verdict came on a suit against the state which had been brought by the American Civil Liberties Union (ACLU) on behalf of 23 local religious organizations, biology teachers and school children. ACLU had asked that the judge overturn the law on the grounds that the act constitutes an establishment of religion, abridges the academic freedom of both teachers and students, and is impermissibly vague, "all in violation of the constitution and laws of the United States".

During ten days of hearings at the beginning of December, ACLU produced a series of religious and scientific witnesses to support its arguments. The former developed the claim that, whatever the wording of the act, they felt its intention to be clearly religious, since the particular set of circumstances which it implied for the origins of the Earth was only compatible, among available descriptions, with the version that appears in the Bible.

Scientists called to testify on behalf of ACLU disputed claims made by creationist groups that conventional evolutionary theory is so full of holes that the alternative hypotheses they support are at least

sufficiently plausible to deserve a hearing in a public school classroom. They also claimed that creation science did not meet the criteria for scientific theories generally accepted by the scientific community, although this was disputed by some of the creationist witnesses produced by state attorney general Steve Clark as part of his defence of the law.

In the end Judge Overton — as had been widely expected — agreed with ACLU's position. But the strength of his criticisms of the supporters of the bill surprised even those who had been expecting a favourable verdict. In a 38-page written opinion, the judge provided a detailed description of the reasons why he found none of the creationists' claims convincing and said that they had admitted that the law was "a religious crusade coupled with a desire to conceal this fact".

The judge criticized the creationists for not taking data and weighing them against alternative scientific data in an empirical way to reach their conclusions. Rather, he said, "they take the literal wording of *Genesis* and attempt to find scientific support for it".

The verdict was, predictably, welcomed with relief by the scientific community. The American Association for the Advancement of Science, whose annual meeting in Washington last week had spent much time discussing the implications of the creationist controversy, issued a statement saying that, in Arkansas at least, "teachers now can get back to teaching science, and students can get back to learning".

Similarly the National Association of Biology Teachers, co-plaintiffs in the ACLU suit, said that it was "gratified" by

Creationism judged a religion, not science

Washington

In his verdict, Judge William Overton said that the evidence presented during the ten-day trial "established that the definition of 'creation science' has as its unmentioned reference the first 11 chapters of *Genesis*", and that "references to the pervasive nature of religious concepts in creation science texts amply demonstrate why state entanglement with religion is inevitable" under the Arkansas law, referred to as Act 590.

"The two-model approach of the creationists is simply a contrived dualism which has no scientific factual basis or legitimate educational purpose", Judge Overton wrote. "The emphasis on origins as an aspect of the theory of evolution is peculiar to creationist literature. Although the subject of origins of life is within the province of biology, the scientific community does not consider origins of life a part of evolutionary theory."

In line with arguments that had been presented by the American Civil Liberties Union, Judge Overton stated that the essential characteristics of science were: (1) that it is guided by natural law; (2) that it has to be explanatory by reference to natural law; (3) that it is testable against the empirical world; (4) that its conclusions are tentative, that is, are not necessarily the final word; and (5) that it is falsifiable. Creation science, he said, as defined in the Arkansas act, failed to meet these essential requirements.

"Some of the state's witnesses suggested that the scientific community was 'close-minded' on the subject of creationism and that explained the lack of acceptance of the creation science

arguments," Judge Overton wrote. "Yet no witness produced a scientific article for which publication had been refused. Perhaps some members of the scientific community are resistant to new ideas. It is, however, inconceivable that such a loose-knit group of independent thinkers in all the varied fields of science could, or would, so effectively censor new scientific thought."

"The methodology employed by creationists is another factor which is indicative that their work is not science. A scientific theory must be tentative and always subject to revision or abandonment in the light of facts that are in-



The next biology lesson is taken from the Book of Revelations....

consistent with, or falsify, a theory. A theory that is by its own terms dogmatic, absolutist and never subject to revision is not a scientific theory."

As he had indicated during the court proceedings last month, Judge Overton made no direct reference in his ruling to the status of evolutionary theory as a science.

David Dickson

the ruling, claiming that "school boards can now resist pressure to include creationism in science classes". Indeed, as a result of the Little Rock decision, a school board in Tacoma, Washington, last week stopped requiring the teaching of creationism in biology classes.

However, the dispute is not going to wither away and the next major test will come later this year in Louisiana, which also passed a law requiring equal treatment for creation science and evolution in its public schools last year. Ms Martha Kegel, the executive director for the state's ACLU chapter which is mounting a comparable challenge to that raised in Arkansas, said last week that she was "elated" not only by Judge Overton's verdict, but by the detailed critique of creation science that he had developed on the basis of the testimony delivered during the Little Rock trial.

Yet the Louisiana case is far from clear-cut. In the first place, the local judge is under no legal obligation to take Judge Overton's verdict into account, since it is a separate jurisdiction.

Second, the Louisiana bill omits some of the detailed provisions of the Arkansas law, for example its stipulation that creation science must include the notion that the Universe, energy and life were created "from nothing", a requirement which several religious witnesses said clearly implied the necessary existence of a creator. ACLU claims that this change only exacerbates the extent to which the bill is unconstitutionally vague; the creationists hope that this revision will remove the basis for some of the strongest objections.

Third, leading members of the creationist movement are likely to take a much more active role in the prosecution of the Louisiana case than they were permitted to do in Little Rock.

Meanwhile in Arkansas the state attorney general has yet to decide whether to implement his previous promise that he would appeal against the verdict if it went against him. And in ACLU, contingency plans are being discussed for Mississippi, in case it is decided that the law should be contested there as well. **David Dickson**

University of London

Separatists emerge

The University of London, parts of which already live with the threat of bankruptcy, now faces balkanization as well. Last week, University College, the largest multidisciplinary college in the university (with 6,000 students) formally asked that it should in future be dealt with financially as if it were an independent university, with its own grant allocation from the University Grants Committee.

The demand is more like gauntlet thrown down before the university's management than a unilateral declaration of independence. Sir James Lighthill, Provost of

University College, nevertheless says that the college would continue to play a full part in the university even if, like Imperial College, it were directly financed.

Especially since last summer's delayed allocation of funds to the London colleges by the court of the university, University College has been a fierce critic of the court's procedures. Last year, Sir James Lighthill won acceptance of the principle that a college's success in obtaining research grants should count — in its favour — in the annual distribution. The precedent for his latest move is Imperial College, which has been directly financed for the past two decades.

The problem now facing the university court, the ultimate authority which shares out funds among the colleges, is tricky. It is certain to regard financial independence for University College as a dangerous precedent. But the court must also be conscious that with the impending retirement of the principal (its chief officer), Mr J. R. Stewart, together with some of his more experienced colleagues, its ability to administer its funds intelligibly may be further impaired.

Discontent about the court's procedures has been simmering since the summer, when the court translated the grants committee's targets for 1983–84 into financial allocations for the present academic year and target student numbers for two years ahead. One difficulty for the colleges is that they are required to adjust to reduced budgets without knowing whether their individual plans for the future will add up to what the grants committee expects of the university as a whole. This gap will be bridged only after the publication (expected next week) of the reports of the committees set up in September to consider the balance of teaching in broad subject areas.

Meanwhile, the non-medical parts of the university have made little headway with reorganization. The announced betrothal of King's College and Bedford College has not led to marriage but to an agreement to associate. The plan for an association between Queen Elizabeth College (the smallest in the university) and Imperial College has been put on ice, partly because Queen Elizabeth College could not accept that the association should be contingent on conditions, such as the provision of new buildings, that could not be satisfied for some time to come.

The late starters have on the whole done best. Chelsea College, faced with starkly reduced numbers and the continuing cost of buying its new site, began the academic year with a draconian plan which entailed the elimination of whole departments, and is now in a position to make substantial economies while softening its plan. And Royal Holloway College, blessed with a huge site 20 miles from central London, is being reluctantly courted by various suitors hopeful that they may be able to turn their city sites into handsome dowries.

Academic censorship

Shadow ahead

Washington

Admiral Bobby Inman, deputy director of the US Central Intelligence Agency, last week dismissed as "somewhat disingenuous" the blanket claims of scientists to scientific freedom in the light of arrangements routinely made with private, corporate sources of funding, and said that the overlap between technical information and national security "inevitably produces tension".

Admiral Inman, who as head of the National Security Agency under the Carter Administration started a dialogue with the scientific community over how to handle potentially sensitive but unclassified research in cryptography, also urged co-operation between scientists and security agencies to find a mutually acceptable relationship "before significant harm does occur which could well prompt the federal government to overreact". He suggested that a potential balance between national security and science "may lie in an agreement to include in the peer review process (prior to the start of research and prior to publication) the question of potential harm to the nation".

The admiral's remarks, delivered to a session forming part of the annual meeting of the American Association for the Advancement of Science (AAAS) in Washington, provoked a strong protest from some of the scientists present.

Professor Peter Denning, for example, professor of computing at Purdue University and president of the Association for Computing Machinery, claimed that efforts to restrict the publication of technical research data reflected a growing protectionist mood in the government which would stifle scientific communication and ultimately prove destructive to the growth of new technologies.

Responding to such concerns, the board of directors of the AAAS later passed unanimously a resolution opposing government restrictions on the dissemination, exchange or availability of unclassified knowledge. Others who took part in the AAAS session, however, accepted that the issue was not clear cut, and that many of the government's concerns were legitimate — even if they had occasionally been executed over-zealously, or had had their legal ambiguity exploited in the past.

Dr Mary Cheh, for example, professor of law at George Washington University, said in a paper on the government control of private ideas that the real question was not whether the government was justified in imposing secrecy on scientific research, but how far its efforts should be permitted to go.

Similarly, Congressman Paul McCloskey presented a paper, read in his absence, describing his legislative efforts to introduce a new bill aimed at clearing up

the ambiguities that allowed the government to prosecute *The Progressive* for publishing an article on how the hydrogen-bomb works, even though it was based on material taken from public library shelves.

As Admiral Inman and others pointed out, however, the debate over the national security implications of scientific research is no longer purely a military question, linking up directly with concerns about the "export" of commercially valuable information through open publication.

"Some of our most carefully nurtured technological advances have suffered from a haemorrhage of foreign technology transfer from international trade — the licit or illicit selling of everything from chips to detailed manuals — to explicit espionage efforts", the session was told by Mr Daniel C. Schwarz, an attorney who, as general counsel to the National Security Agency, had helped draw up the proposed prepublication review procedure for cryptography research.

Admiral Inman also suggested, though emphasizing that he was expressing a personal opinion only, that there were several other fields such as computer hardware and software, lasers, crop projections and manufacturing procedures where publication of certain technical information "could affect the national security in a harmful way".

He also reacted to the charge that the National Security Agency had not provided public proof of its concerns about

the potential damage that could be caused by the publication of the results of cryptography by arguing that such information was often even more sensitive than the basic information itself.

"Nowhere in the scientific ethos is there any requirement that restrictions cannot, or should not, when necessary be placed on science", he said. "Scientists do not immunize themselves from social responsibility simply because they are engaged in a scientific pursuit."

He quoted several other areas, such as controls on genetic engineering research or on the protection of proprietary data, in arguing that there was nothing inherently wrong with an attempt to impose restrictions on science. "Some of these restrictions are common sense, some are federal requirements, some are simply good business and some are good science," Admiral Inman said. **David Dickson**

Polish crisis

Exiled students speak

Paris

Poland's "independent student association" was disbanded last week by order of the Ministry of Science, Higher Education and Technology. According to this decision, the association was disbanded for continuing militant activity after the declaration of martial law. Activists had, it is alleged, continued to distribute leaflets calling for strikes and other protest actions which represented a "flagrant violation of the decree on martial law".

The day after the decree, a group of Polish students, stranded in the West by the declaration of martial law, set up a "coordination group of the independent student association" with a provisional office in Paris. The group intends to work mainly for the relief of academics and students interned in Poland under the martial law regulation. At a press conference in Paris last week, however, it stressed that the current situation, with the threat that oaths of loyalty must be taken by academics and students who wish to continue working in the universities, will prove disastrous for higher education in Poland.

It has therefore asked students throughout the world to press for the liberation of the interned students, and for all intellectuals to take a similar stand concerning intellectual and academic staff. In particular, it called for a boycott of scientific, economic and sporting cooperation with "representatives of Jaruzelski, Moscow and the Eastern bloc" until martial law is lifted in Poland.

One of the speakers at the conference, Miss Anna Krajewska, a philosophy student from Krakow, said that one of the major "sins" of the independent student association had been its participation in a plan to create a new international student

organization independent of all political slant or affiliations.

Recent announcements by General Jaruzelski's military government has accused the association of "striving to create a new anti-socialist student international organization and at the same time to break up the existing international students' association" (a reference to the Prague-based and socialist-oriented IUS).

According to official statements in Poland, the Ministry of Science, Higher Education and Technology (which is at present without a minister) feels sure that members of the former independent student association will "judge correctly" the extremist leaders who "abuse the confidence" of rank and file students. By taking up studies, says the ministry, and "respecting the law", they can be sure of preserving their student status. This is a clear hint that the current process of political "verification" now taking place in Polish industry will be imposed on students before they are allowed to resume their studies. A meeting last week between the rectors of medical academies and political leaders suggests that in medical schools the process will be introduced even more rapidly and strictly. **Vera Rich**

British medical research

Vacancy filled?

Dr J.D. ("Dai") Rees, from Unilever, is for the time being the Medical Research Council's preferred appointment as director of its National Institute for Medical Research at Mill Hill, in suburban London. An appointment has become necessary because Sir Arnold Burgen, the present director, is leaving in the summer to become the Master of Darwin College, Cambridge. A definite proposal has not yet been put to Dr Rees, it is understood.

Dr Rees has been connected with the Medical Research Council for the past two years, since his part-time appointment as co-director of the council's Biophysics Unit based at King's College, London. Recently he has played an important part in suggesting a new management structure for the National Institute, which has an annual budget of £8 million. Part of this proposal, on which senior staff at the institute are being consulted, is that the new director should be advised by a four-member management committee empowered to determine the general pattern of research.

Dr Rees, in his early forties, is a carbohydrate chemist by background who has established a dazzling reputation at Unilever by his work leading to the development of polysaccharides capable of lending physical structure to otherwise fluid materials. The technique is now used commercially in the manufacture of ice cream and of instant desserts.

More recently, Dr Rees has turned to cell biology, including the study of cell movement on surfaces.

Relative prosperity

A gift of \$1 million from Mr Harold McGraw, chairman of McGraw-Hill the publishers, has ensured that work on the Einstein papers can continue to completion. But Dr John Stachel, the editor of the project for the past four years, says that although the gift is enough to ensure continuity, more money will be needed if the staff of the project is to be increased from two (at present) to the desired four or five.

For the time being, work is concentrated on the early years of Einstein's academic life. The intention is to include not merely extant letters and published papers but also ancillary documents. Dr Stachel is pleased to have come across already a letter of commendation from Einstein's eventual colleagues at the Eidgenössische Technische Hochschule (ETH), Zurich (where Einstein went from the Patent Office in Berne) saying, in effect, that although Einstein was a Jew, he was "quite a nice Jew, not like the others".

At this stage nobody is prepared to guess how long the project will take, or how many volumes will eventually be published (by Princeton University Press) although there will certainly be a score of them. Dr Stachel expects to be working on the project for twenty years.

Dr Rees's obvious attractions as director at Mill Hill include his combination of individual experience with his reputation as a scientist and his formidable reputation for getting on well with people.

Among the questions to be settled before an appointment can be offered are an appropriate salary — the director of Mill Hill has traditionally been a physician whose salary has been linked with those of medically qualified academics. There is also a question of the new director's freedom to make appointments of his own.

US agricultural aid

Balance for all

Washington

A report by the General Accounting Office (GAO), the investigative arm of the US Congress, criticizes the ability of US universities to carry out agricultural research and technical assistance relevant to the needs of Third World countries.

The report, recently published in Washington, focuses on the impact of legislation passed by Congress in 1975 which directed the Agency for International Development (AID) to find ways of strengthening and improving the involvement of US universities in solving the food problems of the developing nations. The action by Congress was meant to halt the decline in AID research grants to universities which had taken place in the early 1970s and covers projects totalling about \$400 million.

After a detailed assessment of the various initiatives taken under the new legislation, an amendment to Title XII of the Foreign Assistance Act, known as "Famine Prevention and Freedom from Hunger", the GAO concludes that progress in achieving its goal had been slow and in some cases virtually non-existent.

The debate over the relationship between AID, Third World needs and the US research community has considerable significance for US foreign policy. Increased assistance for agricultural research was one of the recommendations that won universal endorsement — including that of President Ronald Reagan — at October's summit meeting in Cancun, Mexico.

The Reagan-appointed administrators of AID, who see Title XII as providing a firm basis for switching the emphasis of foreign aid towards technical assistance, particularly in agricultural research, claim that parts of the GAO report create an impression "which seriously understates the very substantial progress made during the first five years of Title XII".

However, Mr Herbert L. Beckington, the agency's inspector general, commenting on a draft report, said that in general AID accepts its recommendations and intended to carry them out. And AID deputy administrator Mr Joseph Wheeler told a recent meeting of the Board for International Food and Agricultural Development that plans were already under way to issue a

policy directive clarifying the agency's commitment to the Title XII concept and to make implementation more effective.

AID officials emphasize that the legislation was originally intended to ensure that US universities with a tradition of agricultural research received adequate support to enable them to provide technical assistance to developing nations, a capability which they claim was eroded in the early 1970s as foreign-aid policy switched to commodity and credit agreements. They accept many of the criticisms of the way in which technical assistance programmes have worked out in practice. The GAO investigators, for example, found that many AID field missions distrusted teams sent out by US universities, claiming that members of such teams often lacked relevant experience of the problems they were meant to tackle.

The GAO report also criticizes the AID contract procedures in universities. In some cases, for example, it is claimed that the agency has taken up to two years to approve the award of a contract to a particular university. In addition, it says that the resource registry used to identify the best institutions and individual resources is not up to date, contains inadequate information and is cumbersome to use.

Supporters of Title XII within AID, who feel that their efforts to implement the legislation effectively received only lukewarm support from the Carter Administration, have generally welcomed the GAO report and hope that the new Administration will prove more sympathetic.

David Dickson

Drug regulation in Brazil

Troubles building

Rio de Janeiro

The inauguration last month of a new headquarters for Brazil's National Institute for Health Quality Control underlines the urgent need to bring order to the chaotic system of marketing pharmaceuticals in Brazil. Over the past ten years, Brazil has licensed on average 8,000 new brand names for drugs each year and the country is among the most liberal in the developing world for allowing the exploitation of pharmaceutical products.

Brazil now ranks sixth in the world for pharmaceutical sales, and the market is dominated by the multinational companies which lobby in Brasilia against tighter regulatory measures. Import licences are issued by the Ministry of Commerce which registered US \$5.5 million of "pharmaceutical drug imports" and \$300 million under the category "other pharmaceutical additives" during the first three months of 1981. Total sales last year amounted to almost \$2,000 million, or six times the amount spent for all public health programmes in the country.

Last month Brazil's president, General

Figueiredo, opened a new \$5 million seven-point star-shaped building for the National Institute for Health Quality Control and its director, Eduardo Peixoto, outlined the institute's main role — that of "moderating the free flow of licensing of pharmaceutical products in Brazil". Such steps are clearly needed, but the institute's predecessor, the National Secretariat for Health Vigilance (SNVS), given the responsibility for registering and controlling drugs six years ago, did not set a good precedent. Its Central Control Laboratory functioned for three years with only two technicians, at the end of which time it received its new name — and now after a further three years, a new building.

In the absence of effective controls, Brazil's pharmaceutical industry has gone on expanding despite the general economic recession, with the biggest growth area being psychotherapeutic drugs. Almost all drugs can be sold over the counter in any pharmacy without a prescription and the average consumption per person is several times greater than in developed countries.

One of the most alarming problems is the indiscriminate use of antibiotics. Antibiotics are routinely bought to treat colds, and with six of the ten best-selling drug products being antibiotics there have been many examples of falsification by private laboratories and distributors. The Maerson Laboratory of São Paulo was providing hospitals with "zircillin" and "ampicillin" capsules which in fact contained pure starch. And in Rio de Janeiro, after a series of complaints from consumers, Roche Laboratories discovered that their Bactrim balsam had been the victim of large-scale falsification by distributors and that several Rio pharmacies were selling an adulterated product diluted to half its normal concentration. A further abuse of antibiotics came to light last November when the respected Adolfo Lutz Institute of Biology confirmed the accusation made by the president of the Pharmaceutical Association of São Paulo that the milk sold in São Paulo contained antibiotics used as preservatives.

In a recent report, Dr Carlyle Guerra de Macedo, a consultant to the World Health Organization, denounced the "inefficiency of official controls which permits the marketing of many dangerous drugs". Many drugs are still available in Brazil, even with no prescription, long after they have been prohibited in the United States. And many of the products on sale contain no warnings about the possible harmful effects of abuse. These problems are further complicated by the fact that in many pharmacies the staff are inadequately trained and know little about the products they sell.

The list of faults in the Brazilian way of marketing drugs is extensive, and the revitalized National Institute for Health Quality Control faces a daunting task.

Maurice Bazin

UN technical development

Licence to spend

Washington

The General Assembly of the United Nations has agreed on a new financing system which came into operation at the beginning of the year, aimed at supporting efforts by developing nations to build up their scientific and technological capabilities.

In effect the resolution prolongs for one more year the interim arrangements for carrying out an ambitious plan of action agreed at the UN Conference on Science and Technology for Development (UNCSTD), held in Vienna in 1979. However, the form of the final arrangements is still under discussion.

The money so far received for the plan of action remains far below target. While it was agreed in Vienna, for example, to set up an interim fund under the auspices of the United Nations Development Programme (UNDP) to attract voluntary contributions of at least \$250 million during 1980 and 1981, less than \$40 million has been formally pledged.

The new resolution talks of setting up a financing system which would raise an annual \$200 million over 1983–85. Several UN observers feel that this, too, is wildly optimistic, pointing out that Congress has even deleted the \$10 million the United States was to have contributed to the fund from the foreign aid bill signed by President Reagan in November.

Perhaps more significant than the financial targets, however, has been the outcome of a dispute running since before the Vienna conference over who should control the distribution of the funds.

At UNCSTD the interim fund was established under UNDP, but at the same time a new Center for Science and Technology was created within the United Nations, under a new assistant director-general, whose principal responsibility was to provide secretariat services for an Intergovernmental Committee, open to all UN member states. The committee has had broad responsibility for the policy directions taken by the fund, but despite the strong efforts of some of the more militant developing countries, it was not given direct control of the fund.

During recent negotiations in New York on the resolution to set up a financing system that could be approved by the General Assembly, several of the more advanced developing nations again pressed for the Center for Science and Technology to be given greater responsibility for distributing funds.

Other countries opposed this move, however, on the grounds that if the management of the fund became too much an instrument of the more militant members of the Group of 77, this would further reduce the chances of the fund receiving significant resources from the

developed nations.

Eventually, the latter view prevailed. The financing system approved by the General Assembly will have transferred to it the projects and personnel that now make up the interim fund, with the Intergovernmental Committee involved primarily through a small subgroup which will negotiate with potential donors. The resolution does not mention the centre.

It is hoped in New York that during the first year of these arrangements, definite procedures can be devised for raising donations from both developed and oil-producing countries along the lines endorsed by 20 developing nations at a meeting in Caracas in early October.

Observers in Washington remain sceptical. Although the State Department has offered moral support for the new initiative, Congress eliminated from the foreign aid bill \$38 million which was to have gone to another special fund, the International Fund for Agricultural Development, on the grounds that much of the promised OPEC contribution had failed to materialize. In New York there is more optimism that a pragmatic compromise between political demands and technical needs can eventually be found.

David Dickson

Biotechnology

Going Dutch

Brussels

Dutch biotechnology researchers are awaiting with keen interest a report due early this year which is to define areas deserving future investment. A government-sponsored committee was set up last May under the chairmanship of Professor Schilpeloord of the University of Leiden with the aim of coordinating research in the Netherlands and increasing its emphasis on commercial applications.

The committee, which brings together experts from industry, the governmental applied research organization (TNO) and universities, is a by-product of the Dutch government's drive to stimulate innovation which was launched on the basis of a 1979 white paper which recommended that the government invest in new technologies.

A sum of 4 million guilders (£870,000) a year has been set aside for the biotechnology committee to allocate to selected projects. This is already being spent, with researchers at the University of Groningen the first to benefit. The committee is expected to make recommendations on priority areas for funding which will include not only commercially viable projects but also related needs for developing, testing and producing genetically engineered microorganisms on a commercial scale.

The construction of a laboratory for work at P3 containment level is in progress under the aegis of TNO, and work should be completed in 1983. Whether the new laboratory will be used as soon as it is ready

Cold comfort

Bombay

India has launched its first scientific expedition to Antarctica. A group of 20 scientists left Goa last month aboard the *Polar Circle*, a 600-tonne ship chartered from Norway. The cruise to Antarctica and back is to last 80 days and should include a total of 15 days actually on the ice doing experiments.

The Indian expedition is being led by Dr S.Z. Qasim, secretary to the Department of Environment and former director of the National Institute of Oceanography in Goa.

The Antarctic expedition, which will cost approximately £1.3 million, is one of several major projects of the newly created Department of Ocean Development, directly under Prime Minister Indira Gandhi. The expedition team consists of scientists from ten organizations including the National Institute of Oceanography, the Indian Meteorological Department, the Geological Survey of India and the Indian Institute of Geomagnetism. Two Norwegian scientists are with the expedition to assist in the operation of specialized equipment from Norway.

As well as collecting oceanographic and weather data, the expedition team will be looking for samples containing evidence that India was part of Antarctica before it broke away some 100 million years ago. During the cruise the ship will survey the ocean bed for nodules.

India becomes the third developing country after Chile and Argentina to reach Antarctica — shortly to be followed by China, which is planning to send its first expedition this year. So far there are no plans for a permanent Indian research station in Antarctica.

K.S. Jayaraman

depends on advice from another committee set up last May — the Committee on the Ethical Implications of Genetic Engineering. This panel includes theologians, a professor of ethics, scientists and doctors, and is chaired by a former politician, Mr Oele.

At present the committee is undecided whether to go ahead with a separate report on research at P3 level followed by a general report or to leave the first issue to the final report even though it may mean the laboratory is unused for a while. Research at P1 and P2 levels is currently subject to authorization by municipal authorities but P3 research will be agreed on at national level. Industry in the Netherlands is at present battling against some decisions by municipal authorities which are alleged to be overcautious and restrictive. As an official of the government's science policy department confessed, "in Holland the whole issue is a political can of worms". **Jasper Becker**

CORRESPONDENCE

Data on dioxin

SIR — In discussing the metabolism of chlorinated dioxins, Alastair Hay¹ reviews the epidemiological evidence for an association between soft tissue sarcomas and phenoxy herbicides.

In fact the evidence in favour of an association is much stronger than he suggests. He fails to mention that in addition to the findings of the surveys of two different groups of Swedish agricultural and forestry workers exposed to phenoxy acids and chlorophenols^{2,3}, studies of industrial workers exposed to these substances during manufacture in the United States have also been published. A review of four such studies⁴ showed that 3 of 105 deaths in the workers under observation had been due to soft tissue sarcomas. This compares with 0.07 per cent of deaths in the total population of US males aged 20–84. Since the publication of this review a fourth (non-fatal) case of soft tissue sarcoma has been reported in one of these groups of men⁵. A fifth case has been described in an employee of one of the American firms but it is not clear whether he was under observation as part of one of the published cohorts⁶.

The Finnish study quoted by Alastair Hay has not followed a sufficient number of men for a long enough period to furnish other than weak negative evidence. The study might easily have missed an increased relative risk of five.

The epidemiological data cannot at present distinguish between a risk due to 2,4,5-T itself (or to related herbicides) and one which is due to contaminants of these compounds.

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Beat the clock

SIR — The anonymity of journals' referees has been much discussed. A common nuisance is the tardy referee who sits on papers sometimes for months, and about whom authors can do little. I believe these could be dealt with effectively if the anonymity of referees were maintained for say a month from dispatch to them of the manuscript. After that the editor would automatically pass on the identity of the offender to the authors who could, and usually would, then take up the cudgels directly on their own behalf.

I am sure this simple measure would effect a speedy acceleration of the turnover time of papers in editorial offices. Many of us who are sometimes authors, sometimes referees, would, I am sure, accept this constraint on our latter function in the interests of our former.

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Niche work

SIR — Concerning H.G. Pickles' imagined "very small niche indeed" (*Pharmacology Issues*, *Nature* 1 October 1981, p.355) for *Journal of Ethnopharmacology* compared with *Journal of Cardiovascular Pharmacology* . . . I propose that more people can certainly be directly involved and interested in what plants they can eat or apply therapeutically or in any other useful way (because anyone may pluck, cultivate or look out for plants) than can be directly involved in cardiovascular drugs (because they are not readily available).

All the best to cardiovascular pharmacologists, but please watch out for the relegation to niches. (To be honest, I did think I was writing esoterica when I submitted my first article to *Journal of Ethnopharmacology* . . . but there must be a lot of people out there in a lot of very small niches, because I received 48 reprint requests immediately following publication, and they are still dribbling in.)

HOWARD H. HIRSCHHORN

Arlesheim, Switzerland

Morphic field sports

SIR — The rather intemperate reaction of *Nature* to Sheldrake's hypotheses (24 September 1981, p.245), against which Josephson has protested (*Nature* 15 October 1981, p.594), is in part the result of Sheldrake's own choice of Bergsonian — or Paracelsan — explanations for the effect he postulates, and in part the result of noncommunication between biology and physics.

Had Sheldrake said that the quantum interconnectedness might extend to macrosystems, including biological systems, I do not think that *Nature* would have felt that its virginity was in peril. A model of interconnectedness does in fact flow from Bohm's idea of explication. The experimental agenda is to see how far beyond the subatomic level this patterning extends. If particles correspond to the asteroids and space-ships of a video game, appearing to behave as objects subject to cause-effect, but being in fact virtual displays built up from pulses which bear no translational resemblance to the "display", Darwinian evolution might well be (some would say, "must be") a video game of the same order, appearing to follow simple selection-adaptation principles, as the game-pieces appear to collide, explode and so on — but in fact determined by information from an implicate substrate.

The relevance of interconnectedness to middle-order systems looks like a prime candidate for confirmatory research. If it were confirmed, Sheldrake would be both right in principle and wrong in his postulated mechanism of "morphic fields": viewed in this way, his suggestion is far from absurd.

One awaits with interest *Nature's* reaction to the first book which points out that Bohm's model also blows up the convention of Helmholtzian mind. That suggestion might prove even more alarming than a physics-based neovitalism.

ALEX COMFORT

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Dead-end theory

SIR — In a recent letter Darnbrough, Goddard and Stevely (*Nature* 2 December 1981, p.302) have argued in support of Creationism. They claim that given belief in a God who reveals Himself in the structure of nature they "expect to find evidence consistent with His revelation of Himself". In their view "the fact that all things exist, and show evidence of design and purpose" is the only form of empirical evidence providing support for Creationism.

In their arguments, your correspondents have forgotten that evidence which is merely consistent with a theory does not necessarily support that theory. An illustration of this is that all possible evidence is consistent with the hypothesis that one minute ago a God created everything in the Universe, including our memories. Clearly, for an observation to be consistent with a theory is only a necessary but not sufficient condition for it to provide that theory with evidential support.

A fundamental problem with creation theories is that because the mechanisms proposed are supernatural, the theories fail to provide an explanation of natural phenomena which can be further investigated, developed and understood. In fact, unlike present evolutionary theory, all theories which invoke a supernatural creator are scientific dead ends, failing to lead to new knowledge.

This difference between creation theory and modern evolutionary theory can be illustrated by considering how each handles anomalous evidence. Darnbrough, Goddard and Stevely claim that such evidence tends to be put to one side to await future investigations which will either show the evidence to be erroneous or permit its assimilation into the original theory. They neglect to point out that the development of creation theory through time, unlike evolutionary theory, has been one long retreat from the evidence. In order to assimilate anomalous evidence, numerous *ad hoc* changes have been made to creation theory. Such alterations were made in respect to the age of the Earth, the number of centres of creation, the number of separate creations and catastrophes through time, and the existence of variation within species, to name only the better known examples. Meanwhile, evolutionary theory has not been without its own troubles, as the current debate over possible rates of evolution shows. However, in the past, anomalies have been assimilated into evolutionary theory in a non *ad hoc* manner, leading to the further articulation and development of the theory. This is a major difference between the two theories.

What is being claimed here is that evolutionary theory is explanatory and has a heuristic function which helps our understanding of natural phenomena to grow. Creation theory, however, is a pseudo-explanation which aids us not at all in our understanding of nature and leads us nowhere. Naturally individuals may hold any metaphysical beliefs they wish, but the introduction of the notion of supernatural creation into scientific explanations should be resisted as contributing nothing, or worse, to our understanding of the world.

MALCOLM HADDON

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NEWS AND VIEWS

Europe probes the auroral atmosphere

from Henry Rishbeth

FORMALLY opened by HM The King of Sweden on 26 August 1981, the European Incoherent Scatter programme (EISCAT) has begun its experimental task of probing the auroral ionosphere with ultrahigh frequency (UHF) radar. Next year, the very high frequency (VHF) transmitter should be commissioned and, after some frustrating delays due to transmitter problems, the system will then approach full operation.

The idea of a high-power radar in northern Scandinavia was mooted at the 1969 General Assembly of URSI, the International Union of Radio Science. Following years of planning and negotiations an agreement between national scientific organizations of six European countries, setting up the EISCAT Scientific Association with headquarters at Kiruna (Sweden), entered into force in December 1975. Three of the associates — CNRS of France, MPG of the German Federal Republic and SRC (now SERC) of the United Kingdom — each contribute one-quarter of the funding, the remainder coming from the 'Nordic' Associates — SA of Finland, NAVF of Norway and NFR of Sweden. Total capital expenditure to date is about £13 million.

The incoherent scatter technique¹, envisaged² in 1928 but not seriously pro-

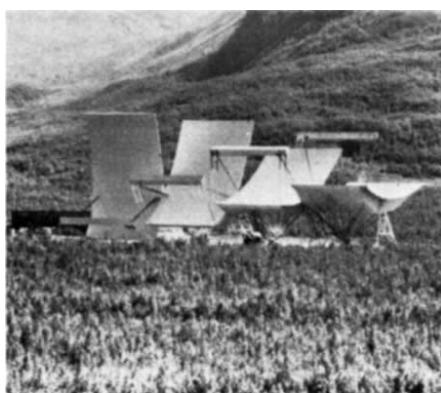
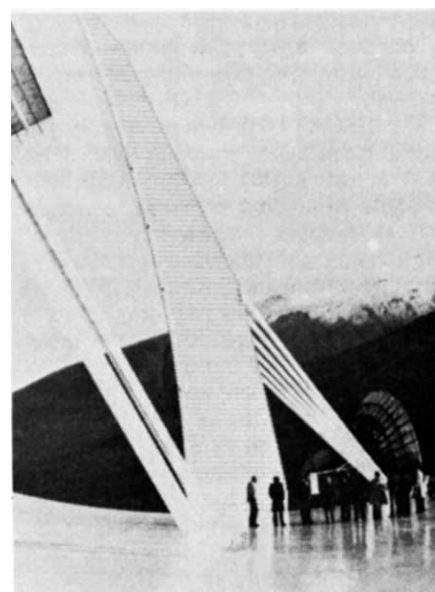


Fig. 2 The EISCAT UHF dish and VHF antenna at Tromsø, Norway.

(EISCAT Scientific Association photographs, taken by Henry Rishbeth.)

posed³ until 1958, was demonstrated successfully in the latter year⁴, and subsequently exploited at a few sites ranging from the magnetic equator in Peru to the sub-Arctic in Alaska. The technique uses a powerful radio beam at a frequency (between 40 and 230 MHz) much too high to undergo the almost mirror-like reflection from the ionosphere that is used in radio communication. The 'target volume' in the ionosphere is defined either by pulsing the



radar beam and sampling the scattered echoes at a selected time delay, or geometrically by using separated, highly directional transmitting and receiving antennas. EISCAT uses both methods in its UHF system, but only the pulse method in its VHF system. Of a peak transmitted power of some megawatts, all but microwatts escape into space and mere picowatts are typically intercepted by the receiving antennas.

The incoherent scatter spectrum (Fig. 1) depends on the spectrum of random thermal fluctuations of plasma density in the ionosphere. Analysis of the spectrum can yield a variety of parameters which (according to circumstances) may include ion density, composition and drift velocity, ion and electron temperature, and ion-neutral particle collision frequency (see ref. 1 and refs therein). Application of well established theory to these data gives several

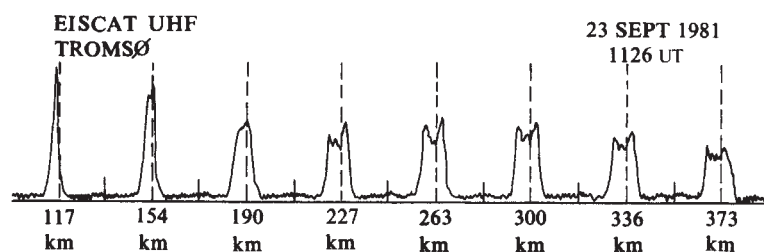


Fig. 1 Early EISCAT data: incoherent scatter spectra taken at eight heights over Tromsø, with approximately 37 km height resolution and 30 s integration. The amplitudes have not been corrected for the inverse-square dependence of scattered power on height. With increasing height, the spectral shape progressively changes from the narrow collision-dominated spectrum in the E region to the broader two-humped spectrum characteristic of the F region. The vertical dashes mark the transmitted frequency, and the vertical bars mark the limits of the 100 KHz passband of each spectrum.

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other ionospheric and upper atmosphere parameters, such as electrical conductivity and current density, electric field, neutral air temperature and wind velocity. Further information on electron density, temperature and drift velocity may be derived from the 'plasma sidebands' flanking the main spectrum, which are excited by photoelectrons or other low-energy particles⁵⁻⁷. In the highly active conditions of an auroral arc, with particle bombardment and large electrical currents, additional spectral features are produced by various plasma waves — the more so in an intensely heated ionosphere such as can be produced by a powerful high-frequency 'heating' transmitter. Incoherent scatter experiments have to be designed within a variety of constraints set by the equipment, the ionosphere and scientific requirements such as height and time resolution. The EISCAT system meets these constraints with a variety of radar techniques: frequency hopping, multi-pulse groups, phase-coded pulses, with digital data processing to match.

The EISCAT UHF system, operating at several frequencies centred at 933.5 MHz (0.32 m wavelength), has three 32 m fully steerable paraboloid antennas, of which that at Tromsø (Norway) is used for transmission and reception and those at Kiruna (Sweden) and Sodankylä (Finland) for reception only. The UHF transmitter is rated at 2 MW peak, 0.25 MW mean power and the VHF transmitter at 5 MW peak, 0.6 MW mean power. The VHF transmitting/receiving antenna at Tromsø (Fig. 2), 120 m × 40 m in size, can scan in the meridian plane by tilting its four panels; its beam can be offset by up to 20° from the meridian plane by inserting phasing cables in the feed system. The USSR plans to build an array for receiving EISCAT VHF signals at a Polar Geophysical Institute site in the Apatiti region.

EISCAT will primarily study the ionosphere, at heights of about 70–2,000 km. The list of measurable parameters shows that the technique can be used to study the upper atmosphere as a whole, not just the ionization. The aim is to build up an integrated picture of the structure, motions and energy sources and sinks of the upper atmosphere. Incoherent scatter observations at low latitudes, complemented by intermittent but more mobile data from satellites, have revealed much about the temperatures, winds and tides — in particular the motions driven by solar UV and X rays.

In addition the upper atmosphere

receives a substantial input of energy from the magnetosphere, mainly at high latitudes. This input is very variable, peaking during auroral substorms when the input of electric currents and high-energy auroral particles sets up atmospheric gravity waves and circulatory winds which carry energy to lower latitudes. EISCAT will study such disturbances at their source, and its electric field measurements in the lower magnetosphere should help to elucidate one major outstanding question: the acceleration mechanisms of auroral particles. The auroral input is superimposed on another high-latitude input, not quite so rapidly varying — the input of momentum from the solar wind — probably through the impulsive connection and subsequent disconnection of the interplanetary and terrestrial magnetic fields as the solar wind flows past the Earth. This process sets up a large-scale circulation of plasma in the magnetosphere and ionosphere, which causes a related circulation of the neutral air above 150 km at polar latitudes.

EISCAT is also being used as a plasma diagnostic to study waves and instabilities in nature's vast plasma laboratory in the magnetosphere. This usage involves cooperation with the GEOS 2 satellite, kept in operation for the purpose, and with the

West German ionospheric heating experiment at Tromsø⁸. The 'plasma physics' aspect of EISCAT's work will be boosted when the VHF transmitter becomes operational.

EISCAT's immediate tasks include cooperation with several other programmes: the NASA Dynamics Explorer satellite, the USSR/French ARCAD 3 satellite, rocket experiments at ESRANGE, Kiruna, and optical interferometric measurements of upper atmosphere winds made from Spitzbergen. There are also investigations of small-scale ionospheric structure and (without the transmitter) radio-astronomical studies of interplanetary scintillations. An experiment is proposed in which UHF signals, transmitted from the Chatanika radar in Alaska and incoherently scattered from the polar ionosphere, will be received by EISCAT. EISCAT will also be used as a stratospheric radar. The move of the Chatanika radar to Greenland, planned for 1982, will intensify the scientific probing of the high-latitude ionosphere, particularly the magnetospheric 'polar cleft'. Meanwhile the processed EISCAT data being accumulated at the Rutherford Appleton Laboratory in the UK, and institutions in other EISCAT countries, will receive keen study from scientists. □

Do all laboratory mice trace back to a single female?

from M.F.W. Festing

In this issue of *Nature*, Ferris, Sage and Wilson suggest that all the 'old' inbred strains of laboratory mice are descendants of one single female who lived some time between 1200 BC and AD 1920 (see p.163). At first sight this is an astonishing claim. Further reflection shows, however, that it is really not so unexpected, and that in view of the maternal mode of inheritance of the mitochondrial DNA (which they analysed) through the egg cytoplasm, the implications for the genetic pool of variation in laboratory mice are not as profound as they would at first appear to be.

What Ferris, Sage and Wilson have done is to study genetic variation in mitochondrial DNA (mtDNA), using eleven restriction enzymes. They sampled wild mice from a wide geographical distribution, as well as nine 'old' inbred strains (for example, BALB/c, C57BL/6 and DBA/2) and five colonies of inbred mice recently derived from wild mice. Among 20 wild mice they found 15 distinct types of mtDNA. All the old inbred mice had identical mtDNA, while four of the five new wild-derived inbred colonies differed from the old inbred strains. They

concluded that the most likely explanation is that all the old inbred mice can be traced back to a single female who could have lived as recently as 1920.

Evidence that wild mice differ in many ways from laboratory mice is accumulating rapidly. The two groups differ in cell surface antigens¹, biochemical markers² and in some types of behaviour³. Several different wild populations have also been shown to carry chromosomal polymorphisms not found in laboratory stocks⁴. Such differences could have arisen as a result of the very different selective pressures on wild and laboratory mice, or as a result of a strong founder effect, or as a result of both these forces. Unfortunately, the historical records are very incomplete and provide little useful information on the domestication of our laboratory mouse stocks.

Mice have been domesticated since at least 1100 BC in China, and our laboratory stocks are believed to be descended from

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these mice following their establishment in Japan, and subsequent importation to Europe by traders in the early nineteenth century⁵. However, mice had also been independently domesticated in Europe, where they were frequently used for auguries and for pharmaceutical purposes.

Many of the old inbred strains may be traced back to the mouse colony established by Miss Abbie Lathrop in Grancy, Massachusetts, in the early part of this century. The origins of these mice are not known in detail, but they included fancy mice imported from Europe, as well as wild mice. Potter⁶ describes this colony as "the American mouse melting pot", but there is very little information on the extent to which wild mice actually contributed to the gene pool. If there were crosses involving the wild mice, it is likely that wild males would have been crossed with tame females, which are far more likely to rear their young successfully in captivity. Thus, such crosses would not have altered the mtDNA, though they would have altered the nuclear DNA of the tame mice. However, not all the old inbred strains are derived from this colony. Strain SWR, one of the strains studied by Ferris, Sage and Wilson, was established from 'Swiss' mice which were not imported into the US until 1926⁷. This would imply either that the Swiss stock became contaminated by American mice, or that the common ancestral female lived some time before about 1920.

Although pedigree records are not available for our mouse stocks, study of other domestic species in which reliable pedigrees are available shows that a single individual often makes a substantial contribution to the development of a breed. Sewell Wright showed that in 1920 55 per cent of individuals of the Shorthorn breed of cattle could be traced back to a single bull, Favourite, born 20–24 generations previously in 1793. Similar close inbreeding was also found in several other breeds⁸. True, breeds of cattle cannot directly be equated with fancy mice. However, there were probably few tame mouse colonies in Europe during the early part of the nineteenth century, and when mouse fanciers did become organized in England in 1895, they were dominated by Walter Maxey, known as "father of the fancy"⁹. Those that found their way into research may well have been passed from one investigator to another as quite small groups, leading to a number of severe population bottlenecks.

If Ferris, Sage and Wilson's findings are not, therefore, too surprising, what are the scientific implications? First, it must be emphasized that what they have shown is that the *cytoplasm* of the old inbred strains may have come from a single female. Few, if any, of the nuclear genes need have come from that female. Thus, their results do not necessarily imply that laboratory stocks come from a narrow nuclear gene pool, as does the Shorthorn breed of cattle. Infusions of new genetic material from wild males, as may have happened in Miss Lathrop's colonies, would leave the mtDNA unaltered. This study will then be of most significance to developmental biologists who are interested in the

relationship between the nucleus and the cytoplasm. In more general terms it also shows that the study of wild mouse populations can be extremely profitable — had Ferris and his colleagues not included wild mice in their studies, they would have concluded that mice are monomorphic for their mtDNA genomes! It is hoped that their work will further stimulate studies of all types of genetic variation in wild mice, with the importation of much more genetic material into the laboratory. This should provide more insight into the genetics and evolution of the mouse, as well as providing many more variants which can be used as tools in the study of all aspects of mammalian biology. □

How black is the Universe?

from Richard A. Muller

ALL but 10^{-9} of the known particles in the Universe are microwave photons of the 3K cosmic background radiation, discovered by Penzias and Wilson in 1965¹, and still the subject of intense study and interest. We believe that these photons are the Doppler-shifted remnant of the thermal radiation from the hot matter of the early Universe. The big bang theory² predicted that the photons would have a Planck frequency spectrum similar to that of the thermal emission from a black (that is, totally absorbing) object; hence the microwave signal came to be known as the cosmic blackbody radiation. True justification for this name came only recently, when the measurements of Woody and Richards³ confirmed that the spectrum followed the general shape of the blackbody predictions.

But the Woody and Richards data also showed a small, statistically significant departure from the perfect blackbody curve. The radiation was slightly more intense than expected at the peak of the spectrum (near 1 mm wavelength) and somewhat less intense in the shorter wavelength region. Woody and Richards were properly cautious about this observation. They state:

"Although there is a statistically significant deviation from a Planck spectrum, there are serious limitations to the statistical analysis where systematic errors are likely. It is clear that another generation of measurement with better accuracy is required before any deviation from a Planck spectrum can be firmly established."

Others have been less cautious. The literature is now full of analysis of the 'observed distortion' by theorists starved for cosmological data. A likely candidate to produce a deviation from the blackbody shape is Compton scattering from free electrons released relatively late in the history of the Universe, during the era of star formation. However, the effect of

Compton scattering is to depress the peak of the spectrum and to elevate the low frequency tail^{4,5}; this signature is opposite to that required. Other possible causes of distortion involve injection of energy in the early Universe, perhaps by early star formation. Unfortunately, most explanations involve so many parameters that one cannot feel confident that a true explanation has been found. Attention is more properly focused on the reality of the distortion.

Measurement of the millimetre region of the microwave radiation is extremely difficult because of interfering thermal emissions from the atmosphere, the Earth and the apparatus itself. Measurements must be made at high altitudes, from rockets, balloons, or satellites. Calibration requires a source with known emissivity and temperature similar to that of the radiation (3K). Accurate calibration is critical; a 27 per cent error could account for the entire distortion reported by Woody and Richards. (The peak would be reduced by the calibration error; the high frequency depression would be accounted for if the temperature of the radiation were 2.7K instead of 2.0K.) Woody and Richards calibrated their balloon-borne instrument on the ground both before and after their flight, and checked the calibration in-flight by observation of atmospheric O₂ emission lines. The check was consistent with the ground calibrations; indeed they have been unable to discover any mechanism which could cause a change in calibration during flight by the amount required to discredit the distortion.

Nonetheless, the in-flight calibration has recently been called into question. H. Pickett, E. Cohen and D. Brinza⁶ at the

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California Institute of Technology measured the pressure broadening of the oxygen lines on which the calibration check depends, and found a value larger than that assumed by Woody and Richards. Ironically, as the Cal Tech group points out, the result is opposite to that needed to explain away the distortion of the microwave spectrum. If we were to use as the primary calibration of the Woody and Richards experiment the oxygen emission measurements with the new widths determined by the Cal Tech group, we would conclude that the distortion in the spectrum is greater than originally reported. It is not clear, however, that the in-flight oxygen measurements provide a better calibration than the ground-based measurements.

Several experiments are being built which could independently test the existence of the distortion. These include not only new spectrum measurements from balloons and satellites, but also high frequency observations of the cosine anisotropy caused by the motion of the Earth; comparison with existing low frequency

anisotropy measurements⁷⁻⁹ allows the shape (but not the overall normalization) of the spectrum to be determined¹⁰. Woody and Richards give sound advice when they urge that we wait for "another generation of measurements" before accepting the deviation from blackness as real. □

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Islet cell precursors are neurones

from A.G.E. Pearce

In a recent report Teitelman and her colleagues¹ describe parallel immunocytochemical studies on the localization, in mouse embryo pancreas, of the peptide hormone glucagon and the four catecholamine-synthesizing enzymes tyrosine hydroxylase (TH), DOPA decarboxylase, dopamine- β -hydroxylase and phenylethanolamine-*N*-methyltransferase. A later study² has added insulin to the list. The presence in a cell of all four enzymes would signify an adrenergic phenotype, of the first three a noradrenergic and of the first two only, a dopaminergic phenotype. With the exception of the decarboxylase, the other three enzymes have been found only in cells of neuroectodermal origin. The results of this elegant study once again bring into focus arguments concerning the derivation of the pancreatic islet cells and hence the whole basis of the APUD concept³, particularly as it concerns the pancreatic and related endocrine cells of the gastrointestinal tract.

APUD is an acronym, standing for amino precursor uptake and decarboxylation, that was first attached in 1968⁴ to a short list of established or presumptive peptide-secreting cells which were situated either in established endocrine glands or diffusely in other regions of the body. These two amino-handling functions were considered to be the most representative ones of seven cytochemical and ultrastructural characteristics possessed in common by the

cells designated by the acronym. Growth of the original list has brought the number of APUD cells to more than 40, if one includes, as one must, the magno- and parvo-cellular neurosecretory cells of the hypothalamus.

Classically the islet cells are regarded as modified intestinal mucosal cells^{5,6}. For around a hundred years they have been considered to arise from stem cells in the pancreatic ducts⁷, thus sharing a common endodermal origin with the cells of the exocrine pancreas⁸. A more modern hypothesis, postulating a neural or neural crest origin for the cells, was first proposed in 1966 as part of the original APUD concept^{4,9}. This view required modification when work with rabbit embryos¹⁰ and avian chimaeras¹¹ showed that the cells of the definitive crest could not be implicated as ancestors or antecedents of the islets, and the concept was then amended to derive them from committed neuroendocrine-programmed precursors arising in the embryonic ectoblast^{12,13}.

The real question is whether in the pancreas the four peptide-producing cells of the APUD series, and in the gut a further fourteen, are descendants *seriatim* and from day to day of multipotent endo-

dermal stem cells, capable of differentiation into enterocytes, Paneth, goblet, duct, acinar and endocrine cells, or whether they arise by transformations occurring in committed neuroendocrine cells. In the former case the enteropancreatic APUD cells cannot be regarded as sensory effectors, and they cannot be components of the postulated third division of the nervous system which the cells are held to constitute. They can only be by-products of an exocrine, mucous, absorptive and duct cell replacement programme. The functional characteristic of the cells, demonstrated cytochemically, and their independently long turnover time¹⁴, have been held to support the second possibility and a neuroectodermal origin, or at least neuroendocrine determination, but morphological evidence has continued to support the unitarian (endodermal) hypothesis¹⁵.

Teitelman and her colleagues¹ have now established, in embryonic mice, that the catecholamine-synthesizing enzyme TH appears in a population of cells in the developing (dorsal) pancreas at day 11. From day 12 to day 14 TH and glucagon can be localized, by serial immunocytochemistry, in identical cells and in up to 40 per cent of all cells containing either antigen. At 14.5 days some of the TH cells are found to contain immunoreactive insulin rather than glucagon².

Expression of the catecholaminergic (dopaminergic) phase is transient; TH is no longer detectable in the glucagon or insulin cells, or in any other cell, after the fifteenth embryonic day. It reappears, in a morphologically distinct islet cell, some time after birth. This observation parallels the transient APUD facility exhibited by enteroendocrine cells in fish¹⁶ and avian¹⁷ embryos. By contrast, the conversion of DOPA to dopamine can be detected in the endocrine cells of the developing mouse pancreas from the eleventh day onwards^{18,19}, and this function continues into the adult state. It may also be observed that Teitelman *et al.* have now provided concrete, rather than inferential, evidence of the presence of the decarboxylase in all the islet cells.

What conclusions can be drawn from their investigations? Teitelman *et al.*¹ formulate their own hypothesis which implies "that all the pancreatic A (glucagon) cells and, conceivably, other pancreatic endocrine cells originate by the transformation of precursors that express catecholamine biosynthetic enzymes". Their current formulation presumably includes the B (insulin) cells as originating in the same manner. What, then, are the implications of this view? "All cells in the periphery that express one or more specific catecholamine biosynthetic enzymes... are of neuroectodermal origin"¹. Do the pancreatic A and B cells constitute the first exception to this rule? That seems unlikely, to say the least.

According to Teitelman *et al.*¹ their data

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clearly "tend to support Pearse's hypothesis" but "full validation of the developmental aspect of the APUD concept would require the identification of the germ layer from which the TH cells of the pancreas are derived". But the germ layer theory has probably outlived its usefulness. The point at issue is not so much whether the APUD cell precursors are initially of endodermal or ectodermal origin, but rather whether they are an irrevocably neuroendocrine-determined self-replicating entity, needing only to effect relatively facile transformations to express functions clearly shown to be related, such as the co-production of amines and peptides⁸ and the switch from one peptide to another, by modification of existing genes (gene splicing)²⁰ or by transcription of closely related genes in place of the original ones.

Significant as they are, the investigations of Teitelman and her colleagues must clearly be extended to include further parallel studies of the association between TH and the other two pancreatic peptides, somatostatin and pancreatic polypeptide (or three if one includes gastrin, which is present in fetal islets). These matters require examination not only in the dorsal pancreatic anlage, where glucagon and then insulin are the first demonstrable peptides, but also in the ventral equivalent where, in the adult, pancreatic polypeptide comes to predominate. We need to know which peptide, if any, is expressed by the morphologically distinct TH-positive cells of the adult mouse islet and, on a broader basis, whether the other peripheral and perhaps likewise the central APUD cells of the pituitary gland and hypothalamus express a transient or permanent dopaminergic function.

Conclusive proof of a neuroectodermal origin has now been established for just two of the enteropancreatic endocrine cells. Confirmation of neuroendocrine status for the remaining sixteen cells can confidently be expected to follow. □

The nature of galactic shells

from Gerard Gilmore

THE outer regions of galaxies are rapidly becoming as mysterious as their nuclei. Kinematic studies have shown that the outer regions of disk galaxies like the Milky Way rotate with the same angular velocity as their inner regions, thus requiring the dominant contributor to the total mass of the galaxy (and the Universe) to be dispersed over a much larger volume than the luminous mass. The nature and true distribution of this 'missing mass' is still unknown. Now it seems that the luminous mass of stars in the outer regions of at least some galaxies is distributed in a much more ordered fashion than expected, with several narrow ripples and shells present at very large distances from the galactic nuclei. In this issue of *Nature* (p.126), Carter, Allen and Malin from the Anglo-Australian Observatory present a detailed study of one such shell, which is 180 kpc (roughly twenty times the distance of the Sun from the galactic centre) from the nucleus of the galaxy NGC1344. Their observations show that the shells are probably normal stars with colours a little bluer than those of the more central regions of the galaxy.

Why then do they form such dramatically sharp features? If the stars currently in the shells have spent their whole lifetimes in the outer regions of the galaxy without perturbation, their velocity dispersion in the radial direction would long ago have smeared out any sharp features in their spatial distribution. The stars must, then, either be young or have had their orbits recently modified. The first possibility was discussed by Fabian, Nulsen and Stewart¹, who suggested recent stellar formation from the gas driven from the galaxy by supernovae blast waves. While young star formation cannot take place in regions of low gas density, Fabian *et al.* showed that shocks can form as the outflowing gas meets the intergalactic medium, thereby producing locally enhanced density and permitting stellar formation. These young stars will, however, fall back into the parent galaxy in the free fall time. Thin shells will be visible only for times short compared with this, or perhaps 3×10^8 years. The shells will therefore be populated predominantly by luminous young stars, which are very blue — too blue, in fact, for consistency with the observations reported by Carter *et al.*, which therefore require another explanation.

One likely possibility is that the shells are remnants of galactic cannibalism. Mergers of galaxies can produce an amazing variety of shapes and structures, possibly including shells. Numerical simulations of the passage of a mass similar to that of a

dynamically cool (disk) galaxy through the disk show a strong circular density wave to propagate outwards, possibly followed by lesser waves². In a recent simulation by Peter Quinn³ of the Mount Stromlo Observatory in Canberra, a spherical and a disk galaxy were merged, and attention was focused on those particles ejected to large distances from the central region. In all such simulations, dynamical friction causes the galaxies to merge after a bumpy meeting. Initially, the disk passes through the main spheroidal component, then falls back in a series of rapidly dying oscillations, each of which ejects some of the disk stars. In this way Quinn was able to generate three or four shells at large distances from the galactic centre, which bear some resemblance to those observed. The stars in the shells are somewhat bluer than the main galaxy as they are preferentially ejected from the younger disk population, in agreement with observations. The shells formed, however, are not as sharp as those seen, nor is it possible to generate the eight to nine separate features seen at different radii in NGC3923. Whether these are fundamental constraints or merely indicate the limitations of the current N-body simulations is not yet clear.

Several examples of galaxies with shells are now known^{4,5}, in some cases with other evidence of anomalous features (loops, jets, active nuclei) which again suggest recent dynamical evolution. Other explanations are, however, possible. For example, the colour differential between the shells and the main body of the galaxy is also consistent with a mild radial decrease of the abundances of the chemical elements heavier than helium, which would not be surprising. The shells would then have to be explained in terms of, for example, internal resonances in the velocity distribution inside the galaxy.

A detailed explanation of the properties of the shells will require both a much more detailed numerical study of galactic mergers, and more specific observations of their properties. David Carter is currently carrying out detailed surface photometry of several examples, to define their luminosity and colour distributions in more detail. While spectroscopy would be highly desirable to define the kinematic properties in detail, it will be extremely difficult. No emission lines have been detected from the shells, and the surface brightness is too low for easy absorption line studies. (The surface brightness

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reported for the outer shell of NGC1344 corresponds to a photon rate of one per arc second per Angstrom every 3 hours with the IPCS on the 3.9 m AAT.) Similarly, no neutral hydrogen 21 cm emission has yet

been detected. Detailed study of the inner shells of this galaxy, while difficult, is possible, and should provide a fascinating insight into the processes of galactic cannibalism. □

Developing synthetic vaccines

from Arie J. Zuckerman

In this issue of *Nature* (p. 158) Dreesman and his colleagues describe a step towards the production of a synthetic vaccine for hepatitis B infection. Using a computer analysis of the amino acid sequence of a major polypeptide portion of the hepatitis B surface antigen they were able to predict hydrophilic regions which would be exposed on the intact 22 nm surface antigen particle and might represent antigenic determinants. Two peptides containing one of these regions were synthesized and found to elicit the production of hepatitis B surface antibody in mice after a single injection.

In the long term the development of synthetic vaccines is an exciting prospect. The ultimate goal is the production of chemically synthesized multivalent vaccines that would replace current bacterial and viral vaccines¹ which can often contain many irrelevant microbial antigenic determinants, proteins and other materials that contaminate the essential immunogen and may cause untoward side effects.

Although use of synthetic peptide vaccine may soon be a reality, the urgent need for protective immunization against hepatitis B virus — it is estimated there are 200 million carriers of markers of hepatitis B virus world wide — is likely to be met in the short term by more conventional vaccines. Progress in their production and towards the creation of synthetic vaccines is reviewed below.

Hepatitis B subunit particle vaccines

The development of a conventional vaccine has been hampered by the failure to grow hepatitis B virus in tissue culture and this has led to attempts to find other preparations which might produce active immunity (see refs 2,3), including the use of inactivated hepatitis B surface antigen purified from plasma of asymptomatic human carriers. Guidelines for the preparation of experimental hepatitis B vaccines were suggested by the WHO Expert Committee on Viral Hepatitis⁴, and the proposed requirements for hepatitis B vaccine were published by the WHO Expert Committee on Biological Standardisation⁵. Progress in the safety and efficacy testing of such vaccines has been rapid. The safety, immunogenicity

and high protective efficacy of two such preparations have been demonstrated by placebo-controlled, randomized, double-blind trials in susceptible male homosexuals who are known to be at high risk of hepatitis B infection⁶ and among healthy volunteer staff from 48 haemodialysis units⁷ and in subsequent studies^{8,9}.

This type of subunit vaccine may, however, suffer from several disadvantages. First, pooled plasma, with high titre of hepatitis B surface antigen (often *e* antigen positive), is required in large quantity from persistent carriers, and it is impossible to characterize each carrier donor on an individual basis. Second, the supply of such plasma may be difficult to secure in the long term and live virus containment facilities are required for production. Third, the manufacturing process is expensive (the reported cost of the vaccine recently licensed is 120–150 US dollars for three doses required for the first course of immunization) and possible extraneous/adventitious agents and other contaminants must be removed. Strict safety testing of the vaccine is required, including tests for residual infectivity of hepatitis B virus in susceptible chimpanzees.

Polypeptide vaccines

To overcome some of these problems, 'second generation' hepatitis B polypeptide vaccines containing hepatitis B-specific antigenic determinants associated with a nonglycosylated polypeptide with a molecular weight in the range 22,000–24,000 and a glycosylated polypeptide with a molecular weight in the range 26,000–29,000 have been prepared and tested for safety, immunogenicity and protective efficacy in susceptible chimpanzees^{10–12}. Although the two major polypeptide species of purified hepatitis B surface antigen of molecular weight 22,000 to 25,000 or 26,000 (variation exists between analyses of purified antigen from different sources), and a glycoprotein of molecular weight 28,000–30,000, have been frequently designated p22 or p23 and gp28, Peterson¹³ considers that these should more properly be designated p25 and gp30, based on the probable true molecular weight of the protein as deduced from the DNA sequence of the viral gene

and the results of amino acid analysis by various techniques.

The advantages of polypeptide vaccine, derived from any source¹⁴, include precise biochemical characterization, exclusion of genetic material of viral origin, exclusion of host- or donor-derived substances and potency¹⁵. Disadvantages of polypeptide vaccines include low yield if strong ionic detergents are used for separation, although the yield has been substantially improved by the use of non-ionic detergents¹⁶.

Genetic biosynthesis

Particularly attractive sources of antigenic material would be prokaryotic cells expressing hepatitis B surface antigen proteins. Cloning of hepatitis B viral DNA and the production of small amounts of surface antigen have been reported by several groups and in significant amounts by Edman *et al.*¹⁷. Expression of hepatitis B proteins in eukaryotic cells has also been achieved in mutant mouse LM cells and in HeLa cells, but sources such as transformed heterotransplantable producer cell lines have not yet been licensed by any National Control Authorities for vaccine production. Expression of glycosylated hepatitis B surface antigen in yeast cells has recently been reported by W.J. Rutter of the University of California, San Francisco, and this is potentially a most important development for large-scale *in vitro* production of an immunogen.

Synthetic hepatitis B peptide vaccines

The feasibility¹⁸ of developing synthetic vaccines was first demonstrated by the identification of the amino acid sequences responsible for immunogenic activity of the tobacco mosaic virus. Such amino acid moieties can be synthesized and, when coupled to carrier protein, will induce the production of neutralizing antibody in experimental animals. In 1976, Langbeheim *et al.*¹⁹ accomplished the neutralization of a bacterial virus with antibody elicited by a synthetic antigen. The first example of successful active immunization against bacterial toxin — diphtheria toxin — using a synthetic antigen has been published recently²⁰. Diphtheria toxin is a single polypeptide chain of 62,000 molecular weight with two disulphide bridges. The loop of 14 amino acids subtended by the disulphide bridge near the NH₂ terminus has been implicated in the toxicity and immunological specificity of the molecule. A synthetic tetradecapeptide linked covalently to two different carriers elicited antibodies in guinea pigs which bind specifically with the toxin and neutralize the dermonecrotic and lethal effects.

Induction of type-specific immunity has also been achieved using a synthetic peptide of *Streptococcus pyogenes* M protein containing only 12 amino acid residues²¹. The immunogenicity of such small peptides points the way to the development of safe vaccines against streptococcal infections which cause rheu-

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matic heart disease, and the efficacy of very small peptides will permit a large portion of the protein molecule to be disposed of, so reducing the chances of eliciting immunological cross-reactions against host tissues. Possible approaches to the development of chemically synthesized hepatitis B vaccines were described a few years ago by several groups of investigators, and current progress suggests that such synthetic peptide vaccines are within reach.

Valenzuela *et al.*²², and subsequently others, identified an 892 base pair region along the DNA strand of hepatitis B virus with the *adw* determinants using cloned DNA fragments, and the full sequence of the 226 amino acids coding for the 23,000–25,000 molecular weight polypeptide of hepatitis B surface antigen was predicted. The corresponding sequence for the *ayw* subtype suggested that it differed in 16 amino acids²³. Employing an unpublished computer program designed by J.E. Kyte and R.F. Doolittle, which has been used to predict the internal and external residues of proteins with known structure, Lerner *et al.*²⁴ chemically synthesized 13 peptides corresponding to amino acid sequences predicted from the nucleotide sequence for hepatitis B surface antigen. Seven out of the 13 free or protein carrier-linked synthetic peptides elicited an anti-peptide response in rabbits. Where used, the carrier protein was keyhole limpet haemocyanin in complete and subsequently incomplete Freund's adjuvant. Antisera against four of the six soluble peptides, ranging from 10 to 34 amino acid residues long, reacted with the native antigen and also precipitated the 23,000 and 28,000 molecular weight major polypeptides of hepatitis B surface antigen. Hopp²⁵ also used a computerized analysis of the amino acid sequences of the surface antigen protein to predict an antigenic site determinant. An amino acid sequence of residues 138–149 was synthesized and examined for its ability to bind antibodies to a mixture of the *ad* and *ay* subtypes of hepatitis B virus. Although the peptide bound only 9 per cent of the antibodies, the maximum binding capacity was not

reached.

Dreesman and his colleagues used a similar technique to predict two hydrophilic regions of the surface antigen molecule. Two cyclic peptides, containing disulphide bonds in the region between amino acids 117 and 137, were synthesized. The two synthetic peptides with amino acids 117–137 and 122–137 were incorporated into several adjuvants including Freund's complete adjuvant, alum, and multilamellar liposomes with and without muramyl dipeptide. Groups of BALB/c mice were immunized intraperitoneally with each of the preparations. Hepatitis B surface antibody was induced 7–14 days after inoculation in approximately 50 per cent of the mice in each group, and in four or five of six mice when the immunizing preparation of the 117–137 peptide was emulsified with Freund's complete

adjuvant. On day 21, the peak levels of antibody decreased in most groups of mice. It should be noted that the antibody response was elicited in mice after a single injection without covalent linkage to a carrier protein.

Thus, synthetic peptides may be used, in due course, as vaccines although mixtures of more than one of the peptides may be required. Of the many questions which remain to be answered, the critical issues are whether antibodies induced by synthetic immunogens will be protective in susceptible non-human primates, and whether protective immunity will persist in chimpanzees and subsequently in man. Nevertheless, it is clear that we are entering the era of antibody engineering, and the prospect of multivalent synthetic vaccines against a variety of microbial agents may well be within reach. □

A problem for the theory of biological structure

from David Eisenberg

AN unexpected result for the structure of the polyoma virus capsid is presented by Rayment, Baker, Caspar and Murakami on page 110 of this issue of *Nature*. It is unexpected because it does not conform to the pattern, proposed by Caspar and Klug in 1962, for the assembly of 'spherical' viruses¹. Their scheme provided a description of highly symmetric structures related to the icosahedron, formed from identical protein subunits, and it seemed able to account for the structure and assembly of a wide variety of structures. The central idea was that of quasiequivalent bonding: in an icosahedron-like, near-spherical shell, each of many subunits can have an environment nearly equivalent (or quasiequivalent in the terminology of Caspar and Klug) to every other subunit. (The importance of this may not seem very great unless you know that it is impossible to arrange more than 60 asymmetric objects in identical environments in a closed shell.) The biochemical significance of quasiequivalent bonding is that each subunit forms nearly equivalent bonds to other subunits. This equivalence of bonding means that the assembly can be simply encoded in the three-dimensional structure of the subunit. It thus becomes easy to imagine how self-assembly of the virus coat might occur, simply through the repeated formation of nearly equivalent, low-free energy contacts.

The Caspar–Klug theory has correlated many observations from electron microscopy and X-ray diffraction, and has been welcome in a field where experimental results are emerging at a rate far greater than principles. Many previous results on structure and assembly have been consis-

tent with the Caspar–Klug ideas. The most striking are the monumental X-ray crystallographic structures of RNA plant viruses from the laboratories of Harrison and Rossmann^{2, 3}. Both studies revealed virus coats that display quasiequivalent bonding of the sort predicted by Caspar and Klug. Thus the new report of decided non-equivalence of bonding of subunits in the polyoma virus coat is particularly startling.

Previous analysis of electron micrographs of the polyoma capsid had shown that the sphere-like shell of a surface is built from 72 clumps of subunits, or 72 'capsomeres'. Twelve of these are surrounded by five identical capsomeres and are thus termed pentavalent. Sixty capsomeres are surrounded by six others and are termed hexavalent. In the Caspar–Klug theory such an arrangement is called $T=7$, where T , the triangulation number, describes the number of subunits and the nature of their quasiequivalence. In a $T=7$ shell, it was expected that there would $7 \times 60 = 420$ subunits; $12 \times 5 = 60$ of these in the pentavalent capsomeres and $60 \times 6 = 360$ of these in the hexavalent capsomeres. What would permit nearly equivalent bonding is that each pentavalent capsomere containing five subunits would be surrounded by five other capsomeres, whereas each hexavalent capsomere containing six subunits would be surrounded by six other capsomeres. This 'five around five' and 'six around six' arrangement results in each subunit having nearly the same environment as every other, and thus being able to

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form nearly equivalent bonds to neighbours.

In their electron density map, Rayment *et al.* find unexpectedly that the hexavalent capsomere is a pentamer, as is the pentavalent capsomere. Thus there appear to be only $(12 \times 5) + (60 \times 5) = 360$ subunits of the major coat protein VP1 in the capsid, rather than the expected 420. The authors buttress their observation by citing studies of the molecular weights of the capsid and VP1 protein which suggest that there are indeed fewer than 420 copies.

Another consequence of the hexavalent unit being a pentamer is that the bonding among coat protein subunits is distinctly non-equivalent. This is illustrated clearly in Fig. 7 on page 114, where the non-equivalence can be seen by comparing panel *c* with panel *d*, and by comparing in panel *d* the various relationships of the five protein subunits of the central capsomere to the six surrounding capsomeres. Figure 7f illustrates that the observed structure can be accounted for by three types of contact between neighbouring pentamers, a much more complicated pattern than the expected single type of contact.

Such an unexpected result, even from the laboratory of a co-author of the Caspar-Klug theory which the result seems to challenge, will no doubt face some scepticism. The refinement method used to obtain the phases for the 22.5 Å resolution electron density map is not the thoroughly tested and trusted multiple isomorphous replacement. In place of heavy atoms to constrain phases, the refinement is constrained only by the icosahedral symmetry and overall dimensions of the capsid. Rayment *et al.* present control calculations that suggest the phase refinement procedure leads to a correct and unique result, and they conclude that "the similarity of the hexavalent and pentavalent capsomeres seen in the electron density map is an intrinsic feature of the virus capsid structure rather than some unaccounted artefact of the refinement procedure". The authors go on to suggest that simian virus 40 and other members of the papova family may all have capsids built of pentameric capsomeres. Some workers in the field of biological structure, while finding all of this intriguing, may reserve final judgement until the study of polyoma structure has reached higher resolution, presumably with the aid of isomorphous replacement.

If the capsomeres of polyoma and related viruses are in fact built all of pentamers, the ideas of Caspar and Klug are in need of some revision. They sought to explain why icosahedral shells had been selected for the design of closed containers built from many identical subunits. They came up with the elegant idea of quasicquivalence: in an icosahedral shell

built from 60T identical units, each unit can have nearly the same environment and hence form nearly equivalent bonds to neighbours. If subunits do not all have similar environments, then the reason for the icosahedral shells is unexplained.

The result of Rayment *et al.* seems to transfer the problem of virus structure and

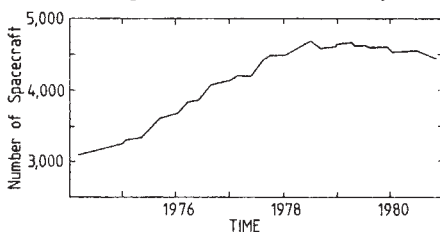
assembly from the realm of completely symmetric structures, such as the quaternary structures of oligomeric enzymes, for which the principles are obvious, one step towards the realm of asymmetric structures, such as the ribosome and the tertiary structures of enzymes, where the principles still elude us.

Spacecraft collisions

from David W. Hughes

THE region of near Earth space, extending to about 5,000 km above the Earth's surface, contains many orbiting satellites and bits of satellites. Collisions between them are inevitable and an estimate of just how frequently this will occur has now been made by L. Schnal and L. Pospíšilová of the Astronomical Institute of the Czechoslovak Academy of Sciences. Their findings are published in a recent edition of the *Bulletin of the Astronomical Institutes of Czechoslovakia* (32, 310; 1981).

First, it was necessary to calculate the number of spacecraft per unit volume of space. Satellites are continually tracked by radar systems in the US and the results published in *NASA Satellite Situation Reports*. The figure below shows how the number of trackable orbiting bodies in near Earth space has varied in recent years.



Between 1975 and 1978 the number was increasing at a rate of about 400 per year but more recently it has remained constant at just over 4,500. Radar sensitivity is sufficient to detect a large majority of objects bigger than 10 cm. Sensitivity is less at high altitudes and for objects below heights of 500 km. The number of small untrackable objects probably exceeds the number of trackable objects by a factor of 2.

Orbital perturbations cause the argument of perihelion and the longitude of the orbit's ascending node to change fairly rapidly and this results in a uniform distribution of spatial density as a function of geocentric longitude. Analysis of the orbital data leads to a value for the spatial density as a function of distance from the Earth and geocentric latitude. The density is highest in the equatorial regions since every satellite must cross the equatorial plane twice per orbit. To calculate the number of impacts per unit time one needs to know the relative impact velocity. Schnal and Pospíšilová came to the conclusion that 7 km s⁻¹ was a reasonable overall

value in near Earth space, agreeing with the previous estimate made by D.J. Kessler and B.G. Cour-Palais (*J. geophys. Res.* 86, 2637; 1978). The computed mean is an averaged value resulting from two sets of collision velocities. Satellite orbital inclinations are unevenly distributed, the majority being due to satellites moving from west to east. One velocity set peaks at about 13 km s⁻¹ and is due to head-on collisions between satellites orbiting in opposite directions. The other set is peaked around the 1–3 km s⁻¹ region and is due to satellites catching each other up.

The authors assume that the mean cross-section of a trackable body is 1.5 m² and that the untrackable objects have a mean cross-section of 0.001 m². The collision frequency, ΔC, is then given by

$$\Delta C = \frac{1}{2} \Sigma A V D^2 \Delta S$$

where *A* is the assumed average cross-sectional area, *V* is the relative velocity, *D* the spatial density and Δ*S* an element of volume. Integrating over all the regions out to a distance of 4,000 km from the Earth's surface results in an average collision frequency per year of 0.017. This is equivalent to around one spacecraft collision per 60 years, the estimated uncertainties being about 50 per cent.

This collision probability has remained reasonably constant over the last few years because the annual number of new launches has remained at about 120 per year. Also, the recent solar maximum caused the atmosphere to expand and this has 'cleaned up' the lower regions by enhancing atmospheric drag and causing the small debris to spiral in quickly. 'Killer' satellite tests and the accidental explosions of rocket motors have also decreased, thus lowering the number of small objects in near Earth space.

A space crash would cause a rapid increase in the number of small orbiting bodies, some of which may be capable of fragmenting another satellite and thus creating even more fragments. But still, with a rate of only one collision per 60 years or so, Schnal and Pospíšilová conclude that inner space is relatively safe. □

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REVIEW ARTICLE

Transcription of *Xenopus* 5S ribosomal RNA genes

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*The accurate transcription of 5S RNA genes when injected into the nucleus of *Xenopus* oocytes or when added to an in vitro transcription system has allowed identification of the DNA sequences and one of the protein factors required for 5S RNA synthesis. Moreover, 5S RNA genes as part of intact chromosomes maintain a transcriptionally regulated state when injected into *Xenopus* oocyte nuclei. A detailed picture of the developmental regulation of 5S RNA gene expression is now emerging.*

DIFFERENTIAL gene expression underlies many of the differences between cell types. Gene expression in turn is controlled at several levels, with transcription especially subject to cellular regulation. There are two general processes which need to be understood in the transcription of eukaryotic genes: one is the nature of the DNA signals and other macromolecular components required for the initiation and termination of transcription of any gene; the other is how transcription is regulated throughout development, that is, what determines whether a particular gene is transcribed at a specific time and in a specific cell.

In the context of differentiation, the most interesting genes are those encoding proteins, as their products determine the cell phenotype. However, genes specifying a small, untranslated RNA molecule, the 120 nucleotide-long 5S RNA component of ribosomes, have so far yielded perhaps the most detailed picture of eukaryotic gene expression and its regulation. 5S RNA multigene families have been best characterized in the South African clawed toad, *Xenopus*, and are the focus of this review (Fig. 1). (For a more general review of eukaryotic gene transcription see refs 1-4.)

In contrast to most eukaryotic mRNAs, which are modified after transcription by the addition of a 5' cap structure and a 3' poly(A) sequence and by the removal of intervening sequences (see refs 4-6 for reviews), *Xenopus* 5S RNA does not undergo post-transcriptional modification^{7,8}; the primary transcript is mature 5S RNA. This has facilitated the development of methods for transcribing purified 5S DNA (DNA containing the 5S RNA genes and flanking spacer sequences). When injected into the nucleus of *Xenopus* oocytes⁹⁻¹¹ (immature, unfertilized eggs), or incubated in a cell-free extract^{8,12-15}, 5S DNA is accurately and efficiently transcribed (Fig. 2). These transcription systems have helped identify the DNA sequences and other macromolecular components required for faithful synthesis of 5S RNA.

Not only are the 5S RNA genes well suited for transcription studies, but they are also amenable to investigations of the regulation of gene expression during development. In amphibians, there are two major classes of 5S RNA genes¹⁶, the 'oocyte type'^{17,18} and 'somatic type'¹⁸. The more abundant class of genes specifies oocyte-type 5S RNA; these genes are transcribed (active) during oogenesis, but are not transcribed in somatic cells^{17,18}. In contrast, the somatic-type 5S RNA genes are expressed in somatic cells as well as in oocytes¹⁸. In the frog *Xenopus laevis*, transcripts from these two types of 5S RNA genes are the same length, but differ in sequence by six nucleotides^{18,19}, which allows the two types to be distinguished experimentally. The regulation of 5S RNA synthesis during development is discussed below.

Expression of 5S RNA genes in injected oocytes

A method for accurately transcribing purified genes was needed to identify the DNA sequences required for RNA synthesis. The transcription of purified 5S DNA after injection into the nucleus of *Xenopus* oocytes was the first example of faithful transcription of an isolated eukaryotic gene (Fig. 2)⁹. In initial

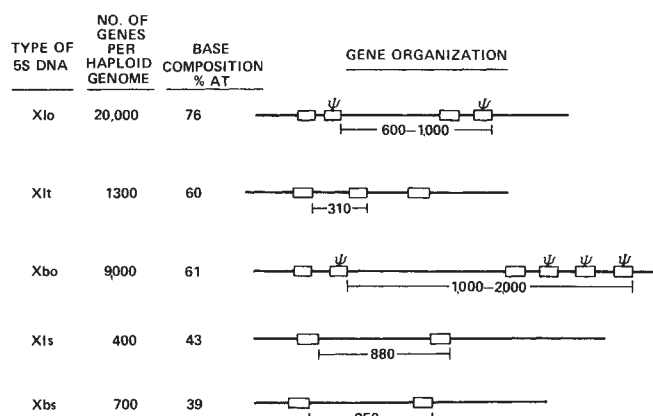


Fig. 1 The organization of *Xenopus* 5S ribosomal RNA multigene families: *X. laevis* oocyte-type 5S DNA (Xlo); *X. laevis* trace oocyte-type 5S DNA (Xlt), the expression of these genes parallels that of Xlo 5S RNA genes⁶⁷; *X. borealis* oocyte-type 5S DNA (Xbo); *X. laevis* somatic-type 5S DNA (Xls); *X. borealis* somatic-type 5S DNA (Xbs). All 5S RNA genes occur in long tandemly repeating arrays. A typical repeat unit of genes and flanking spacer DNA is shown for each family. Open blocks represent 5S RNA genes; those with superscript ψ represent pseudogenes. The base composition of the spacer DNA (% A + T) and the distance between genes or gene clusters (in nucleotides) is indicated. Spacer length heterogeneity is generally due to varying numbers of tandemly-repeating oligonucleotides in the spacer^{23,52,68-71}. Somatic spacer sequences are G + C rich whereas oocyte spacer DNA is A + T rich. The role of spacer DNA in multigene families has been reviewed elsewhere.⁷¹ The DNAs that contain the structural genes for *Xenopus* oocyte- and somatic-type 5S RNAs have been isolated and cloned^{51,52,67,72}. The nucleotide sequence of 5S RNA^{17-19,23,73} and the DNA sequence of a complete repeat unit of each type of 5S DNA has been determined^{13,23,52,68,74,75}. The dominant 5S RNA sequence of each of the five types of *Xenopus* 5S RNA differs from that of the other types by one to several nucleotides¹⁹. The nucleotide sequences of 5S RNA from numerous other organisms have recently been reported⁷⁶. A structure resembling the 5S RNA gene, a 5S 'pseudogene', which differs in sequence from the normal 5S RNA gene by ~10%, is present in every repeat unit of *X. laevis* oocyte-type 5S DNA^{75,77,78} and in some repeat units of *X. borealis* oocyte-type 5S DNA (ref. 23 and L. J. K. and D. D. Brown, unpublished results). In *X. borealis* oocyte-type 5S DNA, the number and relative position of genes and pseudogenes in each cluster is variable (ref. 23 and L. J. K. and D. D. Brown, unpublished results). Pseudogenes can be transcribed by injection into oocytes⁷⁹ or by adding them to an *in vitro* system^{23,48}. There are no pseudogenes in somatic-type 5S DNA⁵². The structural organization of the *Xenopus* 5S RNA genes has recently been reviewed⁸⁰.

experiments, the template consisted of 5S DNA containing many tandem-repeat units of 5S RNA genes and surrounding spacer DNA (see Fig. 1 legend). The subsequent finding that a single cloned 5S DNA repeat can support accurate and efficient synthesis of 5S RNA when injected into oocytes (Fig. 2)^{10,11} demonstrated that each repeat unit contains all the information necessary for its own transcription. This eliminates the possibility that a site at the beginning of an array of tandemly repeating 5S RNA genes is essential for transcription of all the genes in the array. Further delineation of the specific nucleotide sequences and other factors required for accurate transcription was facilitated by the development of an *in vitro* transcription system.

Expression of 5S RNA genes *in vitro*

As purified RNA polymerase does not faithfully synthesize 5S RNA from naked 5S DNA, but transcribes faithfully chromatin containing 5S DNA²⁰, it was clear that either certain structural features of chromatin or chromatin-associated components are required for accurate transcription. Unfortunately, chromatin fractionation has not led to the further identification of factors for transcription. Instead, the identification of transcription factors has come primarily from soluble *in vitro* transcription systems using purified 5S DNA and cell extracts.

The first such *in vitro* transcription system consisted of manually isolated oocyte nuclei, disrupted by gentle pipetting and clarified by low-speed centrifugation⁸. The supernatant accurately and efficiently transcribed cloned 5S DNA (Fig. 2). An *in vitro* transcription system capable of synthesizing 5S RNA can also be made from whole oocytes¹⁴ and from extracts derived from cultured somatic cells^{15,21,22}.

The extracts used in these *in vitro* transcription systems, whether prepared from oocytes⁸ or somatic cells²¹, support 5S RNA synthesis from both oocyte- or somatic-type 5S RNA genes. Thus, although they efficiently and faithfully transcribe 5S DNA, the *in vitro* systems do not show any developmental control. However, using such systems, significant progress has been made in identifying the DNA sequences and other factors required for accurate transcription of 5S RNA, as reviewed below.

Transcription initiation signals

The minimum DNA sequences required for initiation of 5S RNA transcription as well as the sequences which influence the

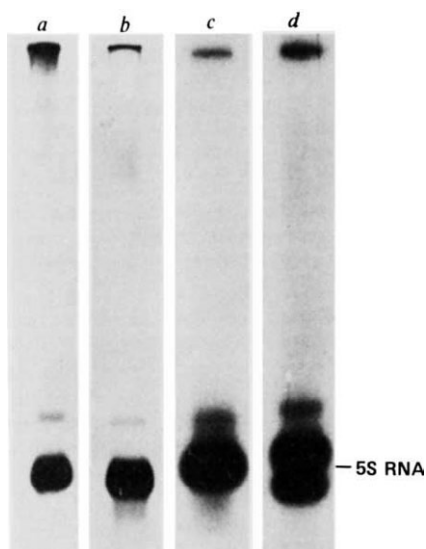


Fig. 2 Polyacrylamide gel electrophoresis of radioactive RNA synthesized after injection of the following types of 5S DNA into *Xenopus* oocytes (a, b) or after addition of 5S DNA to an *in vitro* transcription system (c, d). a, High-molecular weight genomic Xlo 5S DNA; b, plasmid containing Xlo 5S DNA; c, plasmid containing Xbs 5S DNA; d, plasmid containing Xbo 5S DNA. (Reproduced with permission from Brown and Gurdon¹⁰ and Brown *et al.*¹³.)

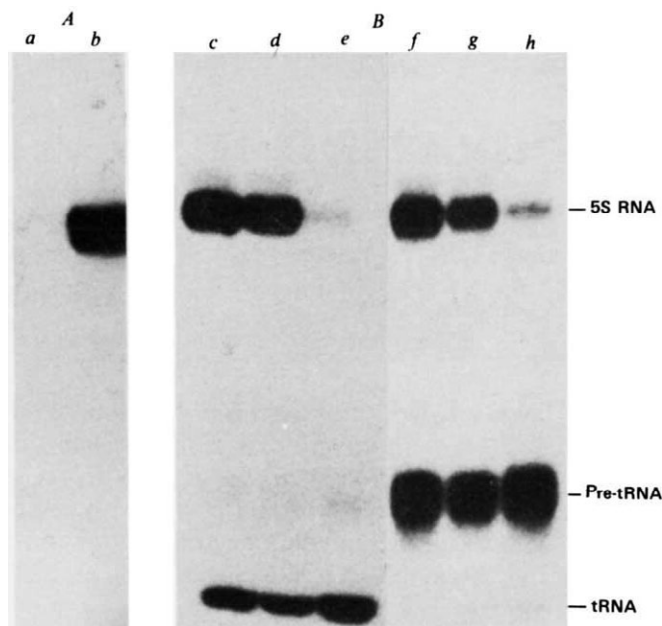


Fig. 3 A, polyacrylamide gel analysis of RNA synthesized in an *in vitro* transcription system containing a *Xenopus* egg S100 with no additions (a), or supplemented with TF IIIA (b). The egg contains very low levels of TF IIIA. This experiment shows that TF IIIA is required for 5S RNA synthesis *in vitro*. (Reproduced with permission from Honda and Roeder³⁷.) B, effect of antibodies against TF IIIA on 5S RNA synthesis. Transcription in an oocyte extract (lanes c–e) or a somatic cell extract (lanes f–h) was performed with a mixture of a cloned 5S RNA gene and a cloned tRNA gene. Lanes c and f, no additions; lanes d and g, plus non-immune IgG; (lanes c–e) or a somatic cell extract (lanes f–h) was performed with experiments show that TF IIIA is required for 5S RNA synthesis, but not tRNA synthesis. The oocyte, but not the somatic cell extract processes tRNA transcripts. (Reproduced with permission from Pelham *et al.*²¹.)

rate or precise site of initiation have been determined by correlating DNA sequence with transcriptional activity. Specifically, DNA sequences altered *in vitro* and recloned have been tested for their ability to support RNA synthesis.

It is known that each repeat unit of 5S DNA contains all the signals required for accurate initiation of 5S RNA synthesis as individual cloned repeat units are transcribed accurately when injected into oocytes^{10,11}. Furthermore, deletion of almost all the A+T-rich spacer sequence preceding the 5S RNA gene does not affect transcription^{23,24}. In contrast to the situation in bacteria, where promoter sequences are located in the 5' flanking region^{25–28}, the minimum sequence required to direct initiation of transcription of 5S DNA has, surprisingly, been shown to lie near the centre of the gene^{29,30}. A series of deletions of the *Xenopus borealis* somatic-type 5S RNA gene which extend varying distances into the gene from the 5' or 3' ends were constructed. These genes have been characterized by DNA sequence analysis and tested for expression in nuclear extracts. It was found that the entire 5' flanking sequence can be deleted without loss of 5S RNA gene transcription^{13,29}. A transcript of approximately the correct size is still produced even when the first 50 nucleotides of the gene are deleted, although transcription is abolished by deletions extending to nucleotide 55 or beyond²⁹. Similarly, initiation is not affected by deleting the 3' flanking region or by deleting the 3' end of the gene as far as nucleotide 83, but is abolished in a gene deleted to nucleotide 80 (ref. 30).

The deletions from each end of the 5S RNA gene identify a central region between nucleotides 50 and 83 which specifies transcription initiation. RNA polymerase initiates transcription at an approximately fixed distance upstream from this control region. For example, when a DNA fragment consisting of the central region (nucleotides 41–87) was inserted into plasmid

DNA, it directed initiation at a site ~41 nucleotides upstream³⁰. In addition, 5S 'maxigenes' were constructed in which 6 or 20 nucleotides were inserted between the control region and the normal site of transcription initiation. In each case, transcription started within the gene rather than at the usual initiation site²⁹.

The precise site and rate of transcription initiation is influenced by sequences at or surrounding the start site^{29,31-33}. Deletions leaving at least 26 nucleotides preceding the 5S RNA gene are unaltered in their transcriptional efficiency³¹. In contrast, deletion mutants with a shorter 5' flanking region are transcribed with decreased efficiencies. Furthermore, deletion of 5' flanking sequences can also allow heterogeneity in the initiation site²⁹. These 5' flanking sequences may include certain oligonucleotides that share homology with the flanking region of other genes transcribed by RNA polymerase III^{13,23}. The exact site of initiation may also be influenced by the preference of this enzyme for starting transcription with a purine residue^{12,23}. Moreover, an examination of initiation sites of transcription by RNA polymerase III reveals that the initiating purine is generally flanked by pyrimidines ($\text{Pyr}^{-1}\text{Pyr}^{+1}\text{Pyr}^{+2}$)¹².

Factors required for accurate transcription initiation

As discussed above, purified RNA polymerase III (the enzyme which transcribes 5S DNA *in vivo*; for review see ref. 34), and 5S DNA are not sufficient for 5S RNA synthesis *in vitro*. To search for other components which might direct accurate initiation, oocyte homogenates have been fractionated. In addition to RNA polymerase III, at least two protein factors are required, one of which has been purified (Fig. 3)^{35,36}. Antibodies raised against this purified protein, transcription factor TF IIIA, inhibit 5S RNA synthesis *in vitro* (Fig. 3)^{21,37}. When TF IIIA is mixed with 5S DNA, it binds to a region in the centre of both oocyte and somatic 5S RNA genes (nucleotides 45-96)³⁵. This region includes that required for initiation of transcription (nucleotides 50-83)^{29,30}. Furthermore, the ability of deleted fragments of 5S DNA to bind TF IIIA correlates with their ability to transcribe 5S RNA *in vitro*³⁸. These results suggest that the influence of the central region on transcription initiation is mediated by TF IIIA.

A protein that shows immunological cross-reactivity with TF IIIA has been isolated from somatic cells²¹. It is unknown whether this protein, or the small amount of TF IIIA which is also present in somatic cells, is the factor required for 5S RNA synthesis in these cells. However, TF IIIA is not required for the expression of other genes transcribed by RNA polymerase III (Fig. 3)³⁵.

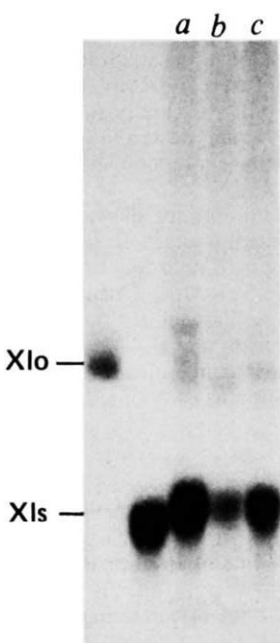


Fig. 4 Polyacrylamide gel analysis of RNA synthesized in *Xenopus* oocytes. 5S RNA genes in somatic nuclei injected into *Xenopus* oocytes continue to transcribe somatic-type 5S RNA, but do not reactivate the oocyte-type 5S RNA genes⁶⁴. Approximately 500 somatic nuclei prepared by lysolecithin treatment of *X. laevis* kidney cells growing in culture⁸¹ were injected into the nucleus of individual oocytes. The RNA from a single oocyte was electrophoresed in each lane of a non-denaturing polyacrylamide gel (lanes a-c). Xlo and Xls 5S RNA markers are indicated. The non-denaturing gel system resolves RNA molecules of the same length but which differ in sequence (for example, Xlo and Xls 5S RNA)⁶⁴. (Reproduced with permission from Korn and Gurdon⁶⁴.)

Transcription initiation signals for other eukaryotic genes

As reviewed above, the sequence which directs the initiation of transcription of the *Xenopus* 5S RNA genes is contained within the gene. Whether this finding is general remains to be determined, but probably depends on the polymerase responsible for transcribing the gene in question. Experiments analogous to those on the *Xenopus* 5S RNA genes show that an intragenic sequence also controls initiation of transcription of the 5S RNA genes in the newt *Notophthalmus*³⁹. Other genes transcribed by RNA polymerase III are controlled in a similar manner to the 5S RNA genes; the control region is within the gene. DNA fragments containing tRNA genes from *Xenopus*⁴⁰, *Drosophila*⁴¹ and *Bombyx*⁴²⁻⁴⁴, in which the 5' flanking sequence has been deleted, can still support accurate transcription initiation when injected into the nucleus of *Xenopus* oocytes or when added to extracts from these nuclei. In contrast, a fragment containing the entire 5' flanking region and the first 30 nucleotides of the *Xenopus* tRNA^{Met} gene, but lacking the rest of the gene, cannot support tRNA synthesis⁴⁵. A single base pair change in the middle of the yeast SUP4 tRNA^{Tyr} gene (position +56) also prevents initiation of transcription⁴⁶. These results suggest that tRNA genes, like 5S RNA genes, contain within the gene a control region which directs transcription. There is also evidence supporting an intragenic control region for the adenovirus VA RNA gene³³. Sequence homologies have been reported between the 5S RNA gene control region and analogous internal regions of the adenovirus VA RNA gene and tRNA genes^{30,33,46}.

The other eukaryotic RNA polymerases seem to require different DNA sequences for initiation of transcription. RNA polymerase II transcribes the genes which specify mRNAs for proteins. Deletion of the 5' flanking sequence of these genes prevents their expression *in vitro* (for review see ref. 4), which implies that DNA signals in the region preceding the gene are essential for transcription by RNA polymerase II. In contrast, the region immediately surrounding the initiation site appears to be critical for gene transcription by RNA polymerase I. Preliminary results indicate that for the *Xenopus* 18S and 28S rRNA genes, the sequence defined by nucleotides -12 to +16 is essential for transcription initiation (B. Sollner-Webb and R. H. Reeder, personal communication).

Transcription termination signals

Termination of *Xenopus* 5S RNA synthesis requires a cluster of four or more T residues in the DNA and is enhanced by an adjacent G+C-rich region^{23,47,48}. These requirements have been demonstrated using mutants. A single base change (T→C), in the cluster of four T residues at which termination of 5S RNA synthesis normally occurs, allows RNA synthesis to continue past this site^{10,13}. Deletions extending into the gene from the 3' end, and thus deleting the T cluster at which termination normally occurs, prevent transcription termination, although the 3' flanking sequence itself can be removed entirely without affecting termination^{30,48}. Moreover, two yeast tRNA^{Tyr} mutations which increase a string of three consecutive T residues in the middle of the gene to five or six T residues, cause premature termination within the T cluster⁴⁶. A similar set of experiments shows that a high G+C content in the region immediately surrounding the termination site increases the efficiency of transcription termination⁴⁸. Prokaryotic termination sequences share with 5S RNA termination sequences T clusters and G+C-rich regions, but also require dyad symmetries that are apparently not needed by the 5S RNA sequences^{23,49,50}.

Developmental regulation of 5S RNA synthesis

To provide for the massive synthesis of proteins during early development, there are at least 1,000 times more ribosomes in an oocyte than in a somatic cell. Two different mechanisms, selective gene expression and gene amplification, are used by the

cell to enhance the rate of synthesis of ribosomal RNA (5S, 18S, 5.8S and 28S RNA) during oogenesis (see ref. 1 for review). Selective gene expression accounts for the synthesis of large amounts of 5S RNA. The 20,000 oocyte-type 5S RNA genes^{51,52} per haploid chromosome set in *X. laevis* are present in all cells, but are only expressed in oocytes, where they account for >95% of the 5S RNA^{17,18}. In contrast, the 18S, 5.8S and 28S RNA genes use gene amplification to synthesize large amounts of ribosomal RNA during oogenesis. *Xenopus* has 450 copies of the genes encoding these RNAs per haploid chromosome set^{53,54}. Disproportionate replication⁵⁴⁻⁵⁷ of these genes in oocytes provides extra templates for RNA synthesis, which are not used after the end of meiosis⁵⁸.

When the oocyte matures into an egg, 5S RNA synthesis stops and remains inactive until blastula formation^{19,59,60}. At this point, somatic-type 5S RNA gene transcription resumes; somatic-type genes account for almost all 5S RNA synthesized throughout the rest of development. Thus, both the amount and type of 5S RNA synthesized varies during amphibian development.

Studies *in vitro* have suggested that the amount of 5S RNA gene transcription is regulated by feedback inhibition by the gene product, as 5S RNA specifically inhibits transcription of cloned 5S RNA genes⁶¹. The mechanism of this inhibition has been determined: 5S RNA binds to TF IIIA to form a 7S ribonucleoprotein particle, thereby competing with 5S DNA for TF IIIA^{37,61,62}. As TF IIIA is essential for transcription of 5S RNA, high levels of 5S RNA inhibit its own synthesis⁶¹. This competition between 5S RNA and 5S DNA for TF IIIA indicates that the amount, but not necessarily the type, of 5S RNA synthesized is determined by TF IIIA.

Several models have been proposed to explain the selective expression of oocyte- and somatic-type 5S RNA genes. In one model an activator, which may be limiting for transcription in somatic cells, has a greater or exclusive affinity for somatic-compared with oocyte-type 5S RNA genes. TF IIIA has certain of the properties of such an activator: for example, it is limiting for 5S RNA synthesis in somatic cell extracts^{21,22}. Moreover, the level of TF IIIA and the rate of 5S RNA synthesis are both high in immature oocytes and fall rapidly when the oocyte matures into an egg^{21,22,35,37}. However, because TF IIIA has only a fourfold greater affinity for somatic- than oocyte-type 5S RNA genes⁶³, whereas the ratio of oocyte- to somatic-type 5S RNA changes 1,000-fold during development⁶⁴, TF IIIA is unlikely to act alone in regulating transcription. The recent discovery of a factor in somatic cells²¹ which has immunological cross-reactivity with TF IIIA raised the possibility that this factor may be involved in regulating 5S RNA synthesis.

Alternatively, chromatin structure may control 5S RNA synthesis. Regions of highly condensed chromatin (heterochromatin) are normally inactive in transcription, perhaps because the DNA is not accessible to certain components of the transcriptional machinery. The 20,000 oocyte-type 5S RNA genes per haploid genome of *X. laevis* are located in blocks of ~1,000 near the telomeres of the long arm of most or all of the 18 chromosomes⁶⁵. The telomeres are thought to be heterochromatic in somatic cells⁶⁵; however, they may not be condensed in oocytes, where the oocyte-type 5S RNA genes are transcribed. This association of the oocyte-type 5S RNA genes with the telomeres therefore offers a possible explanation of their developmental regulation. In support of this model, *X. laevis* liver cells labelled in organ culture or kidney cells in culture, do not normally synthesize oocyte-type 5S RNA^{64,66}. However, in one tissue culture cell line some 5S RNA genes are

translocated to a novel site adjacent to the nucleolar organizer⁶⁵. In this cell line, 10–20% of the 5S RNA synthesized is oocyte-type⁶⁶.

Other models for the developmental control of 5S RNA synthesis include a repressor–operator system analogous to that found in prokaryotes, and changes in the DNA methylation patterns during development. To differentiate between these models, a system is needed that mimics the regulated synthesis of 5S RNA and can be manipulated experimentally. There is no evidence of transcriptional regulation of 5S DNA purified from *Xenopus* or cloned into plasmids. When injected into *Xenopus* oocytes^{10,11}, or added to an *in vitro* system prepared from either oocytes⁸ or somatic cells²¹, both oocyte- and somatic-type 5S RNA genes are transcribed. A likely explanation is that the proteins which are normally associated with the DNA in a eukaryotic cell are absent from cloned DNA.

One study of the selective expression of oocyte and somatic 5S RNA genes during development, without using isolated DNA or cloned genes, has involved the transplantation of somatic cell nuclei into *Xenopus* oocytes⁶⁴. When nuclei derived from erythrocytes or tissue culture cells are injected into oocytes from some frogs, they continue to synthesize somatic-type 5S RNA, but do not express the oocyte-type 5S RNA genes (Fig. 4)⁶⁴. This finding leads to two important conclusions. First, 5S RNA genes as part of intact chromosomes behave differently from purified 5S RNA genes with respect to regulation of transcription. Second, the developmentally regulated transcription pattern of 5S RNA genes can be maintained when genes are transferred from one cell to another as part of chromosomes. In specific conditions, including treatment of nuclei with 0.35 M NaCl before injection (a condition which removes some nonhistone chromosomal proteins, but not histones), the oocyte-type genes can be reactivated⁶⁴. Similar results have been obtained using isolated *Xenopus* chromatin²². The ability to manipulate 5S RNA genes in chromosomes in such a way as to alter their transcriptional capacity should provide means of identifying factors involved in controlling their expression.

Surprisingly, certain frogs have oocytes in which the oocyte-type 5S RNA genes of injected somatic nuclei are expressed (activating oocytes)⁶⁴. When extracts from such activating oocytes are injected along with somatic nuclei into non-activating oocytes, these genes are then expressed (L.J.K. and J. B. Gurdon, unpublished results). NaCl treatment of somatic nuclei or addition of extracts from activating oocytes may exert their effect by respectively removing or adding specific factors which control 5S RNA transcription⁶⁴. Alternatively, they may act by altering chromatin structure and in so doing influence the ability of factors already present in the cell to regulate 5S RNA synthesis. With our present understanding of the DNA sequences and factors required for accurate transcription of the *Xenopus* 5S RNA genes, and the ability to modify selectively that transcription, a complete molecular description of the developmental regulation of 5S RNA expression may be in sight.

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ARTICLES

After the deluge: Mediterranean stagnation and sapropel formation

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In an East Mediterranean marine core, the upper sapropel begins soon after the start of a global event—a very heavy precipitation which occurred in the equatorial latitudes during the late Glacial–early Holocene. This heavy precipitation in Africa, channelled by the Nile River across 35° of latitude, produced a low-salinity surface layer in the East Mediterranean. In this confined basin, with high bottom salinity, the steep salinity gradient stratified the water column. The stagnant bottom waters triggered the sapropel formation. Cretaceous sapropels in the tropical oceans may result from the same chain of events in warm, humid climates, with contrasting wet-and-dry seasonal rhythm.

THE periodic sedimentation of black, pelagic muds rich in marine organic matter (sapropels) has been described extensively in Quaternary and Neogene cores of the East Mediterranean Sea^{1–6}.

Sapropel formation involves the establishment of a steep vertical salinity gradient in the water column, initiated by highly saline bottom waters¹ and completed by a low salinity surface layer^{2,3,5,7,8}, which prevents thermohaline convection and triggers a stable density stratification^{1,3,4}. Stagnant bottom waters are rapidly depleted of oxygen, and this reducing environment preserves the pelagic organic matter. The epipelagic fauna is unusual in abundance, assemblage^{2,7–9} and isotopic composition^{5,7,10–12}, the mesopelagic fauna is normal and the benthic fauna almost disappears^{2,8,9,13}. Sapropels occur mostly during Quaternary warming phases, but also during some cool isotope stages^{3,5,7,8,14}.

As a key to stagnation control, several hypotheses on the origin of the low salinity surface layer have been formulated. The Eurasian ice-sheet meltwater hypothesis^{2,3,5,11,15,16} is rejected by our ¹⁴C data of the most recent sapropel, 11,760–7,950 yr BP: its flushing into the Mediterranean, through the Bosphorus, ended too early, around 13,500 yr BP (ref. 17). It may have lowered the sea surface salinity, but that did not trigger stagnation.

The local Mediterranean increased rainfall hypothesis^{1,2,5}, is not supported by pollen data (Fig. 1): in Greece and south-west Turkey, the warming trend started around 10,600 BP, and was probably accompanied by a small increase in moisture which became significant only after 8,200 yr BP (refs 18–20). Further south, in Crete²¹, moisture increased around 9,500 and after 10,000 yr BP in Syria²², where warming occurred at 11,500 yr BP.

Table 1 Upper sapropel ^{14}C dates in Mediterranean cores

Ref.	Core	Sediment	Depth (cm)	Age (^{14}C yr BP)
72	Alb-194	Sapropel mud	9–17	8,330 $\pm$ 130
73	V10-64	Carbonate in sapropel:	23.5–28.5	
		coarse fraction		8,700 $\pm$ 1,000
		fine fraction		7,400 $\pm$ 200
		organic matter		8,400 $\pm$ 250
74	24M067	Sapropel		7,900 $\pm$ 170
75	OT 26	Varved layer	174–178	8,210 $\pm$ 185
	OT 25	Under finely laminated layer	core basis	9,640 $\pm$ 150
14	P6510-4	Organic ooze above sapropel	21–28	6,575 $\pm$ 160
		Sapropel (50–63 cm)	51–60	8,215 $\pm$ 160
		Hemipelagic mud below	101–110	10,475 $\pm$ 140
This work	KS 52	Nanno-ooze above sapropel-S1	15–20	6,659 $\pm$ 89 CRG 97
		Nanno-ooze below sapropel-S1	35–40	15,945 $\pm$ 196 CRG 98
		Nanno-ooze	45–47	20,011 $\pm$ 323 CRG 99

appear during sapropel deposition and precede ~ 600 yr the deposition of the intercalated oxygenated ooze, during which the maximum $\delta^{18}\text{O}$ is reached. This interval of high values is synchronous with the Younger Dryas cold event (11,160–10,000 yr BP).

(3) A second large, rapid decrease of 3.75% before deposition of the younger part of the sapropel, until minimal values are reached between 8,000 and 6,000 yr BP. A subsequent increase of $\sim 1.4\%$ leads to present day (average) values, which appear in the upper 10 cm.

^{13}C depletions correlate with minimal $\delta^{18}\text{O}$ values at 28.5 and 23.5 cm, but this species displays a rather strong non-equilibrium fractionation²⁸.

Palaeoclimatic implications

The East Mediterranean basin thus experienced a slow and complicated isotopic transition from glacial stage 2 to postglacial stage 1. The overall 3.75% $\delta^{18}\text{O}$ variation is more than double the 1.7% normal difference between glacial stage 2 and present-day values. The 2.05% additional difference is too large to signal only a temperature increase, which would be an excessive $+10^\circ\text{C}$. A 2–3 $^\circ\text{C}$ increase is generally accepted in the Mediterranean during Termination I^{5,8}, accounting for a $\delta^{18}\text{O}$ of -0.7% . The remaining 1.35% has to be accounted for by fresh water influx forming a low salinity surface layer^{5,7,10,11}.

Moreover, both ^{13}C and ^{18}O isotope depletions occur rapidly before sapropel deposition. A simple calculation shows that the isotopic composition of the fresh water entering the Mediterranean has to be higher than -10% . This low estimate suggests that precipitation and river discharge are the dominant source of the surface brackish water layer, and not ice sheet meltwater, which would have an isotopic composition around -30% . River floods started around 12,500 yr BP (second half of the first decrease), waned around 11,000 yr BP, and reappeared immediately after 10,000 yr BP.

The earliest 2.5% $\delta^{18}\text{O}$ decrease (45–30 cm), dated 20,000–12,600 yr BP, is accounted for by three elements: part of the 1.7% global isotopic effect, the 2–3 $^\circ\text{C}$ temperature rise, and some salinity decrease due, until 13,500 yr BP, to meltwater influx through the Bosphorus. Partial stagnation developed, resulting in the grey protosapropel layers. Sapropel formation

was triggered, after a 230-yr time lag, by the second, sharp, highest 1.5% $\delta^{18}\text{O}$ depletion, (30–28 cm) between 12,600 and 11,700 yr BP, entirely a salinity effect due to heavy floods.

The Nile River is the only contender for such a discharge. Before the Aswan High Dam construction, Nile flood water was partly carried eastward along the Israeli coast, where it flowed as a 15-m thick surface layer, decreasing the salinity from its normal values of 38.8‰ to 31.8‰ in September²⁹. Local rainfall from December to March decreases the salinity only to 36‰ (ref. 30). The Nile flood does not seem to exert a temperature effect³⁰. Present-day mean weighted isotopic values of precipitation at Entebbe, Uganda, is -2.91% (ref. 31). The freshwater discharge becomes brackish when mixed by winds, currents and internal waves with the seawater. The Nile origin of the brackish surface layer accounts for the frequently observed east–west gradient of decreasing stagnation and surface productivity in the sapropels. A 10% decrease (from 38‰ to 34–35‰) of the surface salinity is sufficient to induce water stratification. Today, stable stratification is observed seasonally in the open tropical oceans where the freshwater discharge of large rivers spreads as a brackish surface layer (<50 m deep), sometimes as far as several hundred kilometres. Stratification from monsoonal discharge occurs in the Andaman Sea, caused by the Irrawaddy³², off the West Coast of India with the runoff from the Ghats³³, and from non-monsoonal (zenithal) discharge, in the West Equatorial Atlantic under the influence of the enormous Amazon flow, which sends isolated lenses of brackish water as far as Barbados³⁴. In all these cases, a 2–4‰ vertical salinity gradient in the upper 50 m is responsible for the density stratification, whereas the generally warm temperature has little effect on the pycnocline.

The present day Amazon discharge (75 times the Nile), would cover the entire Mediterranean Sea with a 3-m deep freshwater layer in one year³⁴. If the late Glacial Nile discharge was 2.5 times larger than today, like the present Zambezi, it could have accumulated a 25-m deep freshwater surface layer over the Levantine and Ionian basins in only 15 yr. The lags we see in the core between the two sets of heavy floods and sapropel incipience are 230 and 800 yr.

The very rapid 3% $\delta^{18}\text{O}$ increase from 28 to 26.5 cm (11,000–10,000 yr BP) is a result of the cumulative effects of the Younger Dryas glacial buildup, the colder temperatures, and the dramatic decrease of Nile floods, resulting from the simultaneous period of aridity in equatorial Africa. This arid episode is clearly seen in many African low lake levels and pollen diagrams (Fig. 1). It occurred even though the very high incoming summer insolation²³ could have been expected to promote heavy tropical rainfall. The insolation effect in this case is overrun by the atmospheric cooling of high-latitude origin. Colder temperatures in the Mediterranean area are reflected in the Macedonian pollen diagram (zone Y3)¹⁹.

The sharp 3% $\delta^{18}\text{O}$ depletion from 24.5 to 23.8 cm took place in about 300 yr. It is much too large and rapid to be caused solely by ice melt and warming of the Younger Dryas–Preboreal transition²⁷. It signals a salinity lowering due to resumption of heavy Nile floods after 10,000 yr BP. They correlate with the African main rainy period from 10,000 to 8,000 yr BP which builds the highest lake levels around 9,500 yr BP (Fig. 1).

The second half of the sapropel is a consequence of these floods. The time lag between the high floods and sapropel incipience is ~ 800 yr, which is much longer than the previous one, because the floods discharged over a much more saline sea surface.

In the shallower East Mediterranean core GA32, the bottom-to-surface $\delta^{18}\text{O}$ gradient increases suddenly at two levels, suggesting a two-step flooding sequence¹². The isotopic variation related to salinity change is also 1–1.5‰ here.

Although high nutrient input and planktonic productivity are associated today with the arrival in September of the tongue of Nile flood water off the Israeli coast^{30,31,35}, one should not argue that the low $^{13}\text{C}/^{12}\text{C}$ ratios in surface waters are caused by a higher nutrient input. (Each plankton net haul yields 3 cm³ of

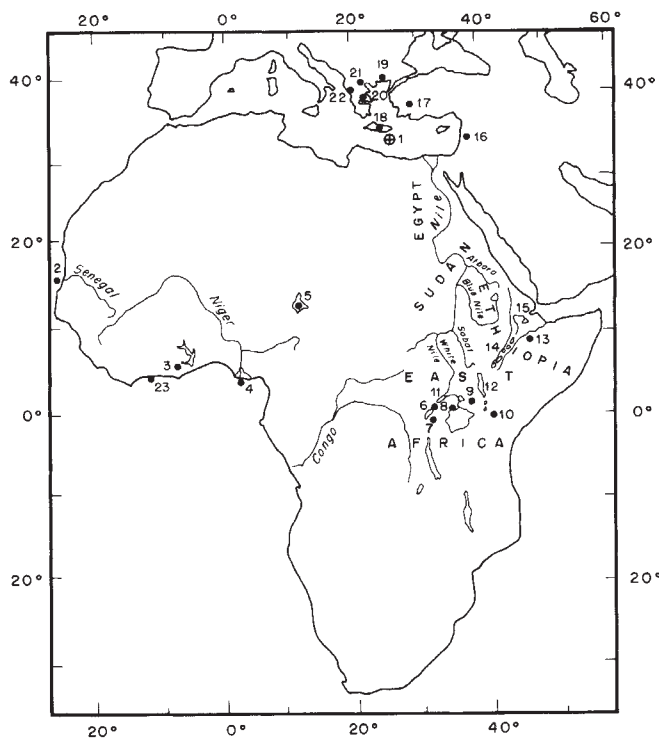


Fig. 2 Location of core and mentioned sites. 1, Core KS52. 2, Core off the Senegal River mouth. 3, Lake Bosumtwi, Ghana. 4, Core off the Niger River mouth. 5, Lake Tchad. 6, Ruwenzori, Lake Mahoma. 7, SW Uganda, Muchoya Swamp. 8, Lake Victoria, Pilkington Bay. 9, Cherangani Hills. 10, Mt Kenya, Sacred Lake, Lake Rutundu. 11, Lake Mobutu Sese Seko (Albert). 12, Lake Turkana (Rudolf). 13, Mt Badda. 14, Ethiopian Rift Lakes. 15, Afar Lakes. 16, Ghab Valley, Syria. 17, Sogüt, Anatolia. 18, Aghia Gallini, Crete. 19, Tenaghi Philippon, Macedonia. 20, Xinias Lake. 21, Edessa. 22, Ioannina. 23, Ivory Coast littoral.

plankton in August, 30–35 cm³ in September; the yearly average is 4.7 cm³ and the spring bloom reaches only 12.5 cm³ (ref. 31). In the Indian Ocean, the $\delta^{13}\text{C}$ of shallow dwelling foraminifera is not related to nutrient supply of upwelling origin³⁶. High primary productivity might even be the cause of a higher $\delta^{13}\text{C}$ of the ΣCO_2 at the same depths (J. C. Duplessy, personal communication). Thus, the depletion we measure in core KS52 are related to the influx of organic rich freshwaters (with a low $^{13}\text{C}/^{12}\text{C}$ ratio of the ΣCO_2) of continental origin. These low surface ratios result in an intense vertical $\delta^{13}\text{C}$ gradient in the water column.

Pollen

The upper sapropel pollen spectrum (pollen sum 356) (around 9,500 yr BP) predominantly reflects a Mediterranean (48.2%) and warm temperate (6.5%) vegetation. It is very similar to the earliest Holocene in Macedonia (Zones Z1 and 2, 10,300–9,000 yr BP)¹⁹. An open (herbaceous: 45.4%) oak (31.1%) pine (15.1%) forest occupied the Greek and Turkish Mediterranean shores, and broad-leaved trees (6.5%) requiring more summer moisture grew higher on the slopes. Littoral or desert halophytes (11.1%) were more abundant here than in Macedonia. The cold steppe *Artemisia* is hardly present (6.0%). The pollen is transported by wind to the sea surface, then dispersed by currents as a pelagic element³⁷. Well specified Nilotic pollen³⁸ is absent: the Nile water does not appear to carry pollen further than its sedimentary load; they are only found in Nile silt³⁸. The low density characteristic of the Nile water is thus preserved far beyond the silt and pollen deposition limits. The geological implication is that bottom waters in a deep or shallow basin may become anoxic under the influence of fluvial discharge even if the local climate reflected by pollen cannot feed such rivers, and even where clastic sediments do not reach. In fact, bottom anoxicity may occur whatever the lithology of the depositing

sediment, provided distance from the continent is not too great. Moreover, this limiting factor can be enlarged in a globally warm climate like the Cretaceous, during which weak winds and sluggish ocean currents do not enhance water mixing.

Late Glacial–early Holocene equatorial deluge

During the last Glacial maximum (19,000–13,000 yr BP), extreme aridity spread in the tropics. It was immediately followed, during the late Glacial–early Holocene (Alleröd–Preboreal–Boreal), by an equatorial heavy rainfall period of global scale, recorded by pollen, in northern South America (Guantiva Interstadial)³⁹, the Galapagos^{40,41}, most probably New Guinea⁴², with extensive wet Lower Montane *Nothofagus* forest, Sumatra⁴³, and the Indian Ocean and Africa. Raised lake levels are also conspicuous. It was the wettest period of the past 20,000 yr.

The formation of the Mediterranean sapropel results from a link (the Nile River) across 35° lat. between this climatic event in equatorial Africa and the marine hydrology. The relevant data are shown in Fig. 1.

In Africa, from west to east, and within 10° N of the Equator, the highest precipitation is recorded between 12,500 and 8,000 yr BP. In West Africa, data are from the southern drainage area of Lake Tchad⁴⁴, the Ivory Coast mangrove peat⁴⁵, the level of Lake Bosumtwi in Ghana⁴⁶, and the Niger River discharge⁴⁷. North of 10° N, the onset of the rainy period is delayed. The mangrove along the Senegal River lower course (17° N) thrives after 9,600 yr BP (ref. 48), and the high level of Lake Tchad (13–15° N)⁴⁹ and its wettest vegetation⁴⁴ occur after 8,500 yr BP.

In East Africa, where the Nile watershed is situated, reinterpreted pollen data from the mountains, 3°S–2°N, correlate aridity before 12,000 yr BP with a cool climate, and moisture thereafter with a warm climate^{50,51}. On the Ugandan (Ruwenzori, Kigezi^{52–54}) and Kenyan (Mt Kenya, Cherangani Hills^{55,56}) mountains, the shift towards a wetter and warmer vegetation

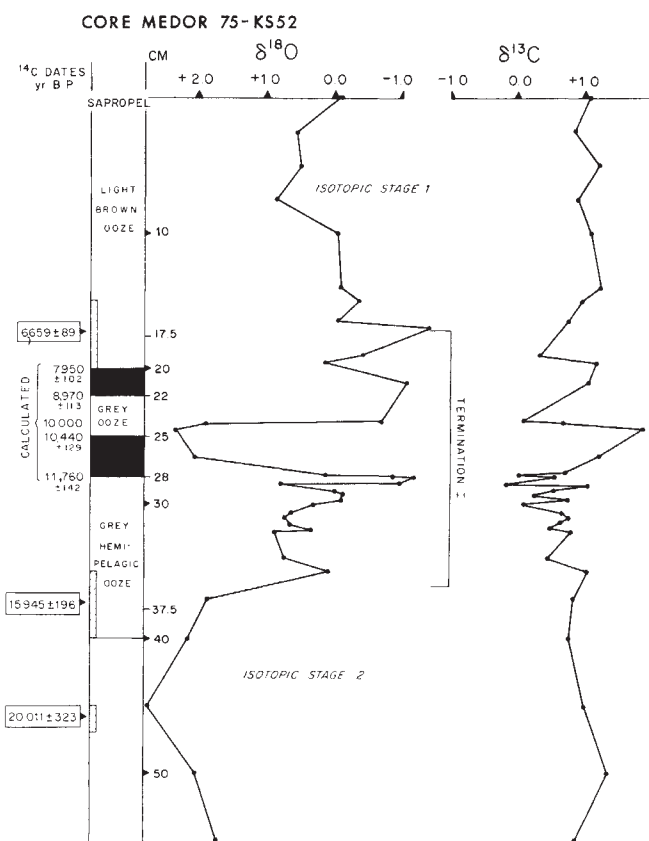


Fig. 3 Sapropel age, ^{18}O and ^{13}C isotopes stratigraphy in core KS52.

belt, of different types according to the altitude of the site, started as early as 12,700 yr BP and developed around 10,800 yr BP. The warmest and wettest local vegetation is usually installed around 8,000 yr BP.

In the lowlands around Lake Victoria, the moisture increased after 12,500 yr BP, and the wettest climate is signalled between 9,500 and 7,000 yr BP by the vegetation, the lake level and its sediments⁵⁰. A widespread dry spell, synchronous with the Younger Dryas, is recorded between 10,800 and 9,500 yr BP in the low levels of Lakes Bosumtwi, Tchad and Victoria, as well as in the vegetation around Lake Victoria and on Mt Kenya.

Before 12,000 yr BP, the Main Nile was dry most of the year, the meagre summer flow fed only by the weak Ethiopian monsoon. After 12,000 yr BP, the East African bi-seasonal rainfall was higher and the White Nile became a very large permanent river, well regulated by the lakes^{51,57,58}. Its catchment area was expanded from 9,500 to 7,500 yr BP by the inclusion of the much higher, larger Lake Turkana⁵⁹.

Wetter conditions occurred on the southeastern Ethiopian Plateau at Mt Badda, after 11,500 yr BP or possibly only 9,500 yr BP (ref. 60).

Drained by the Blue Nile and the Atbara, the northern Ethiopian Highlands (10–13° N) are inaccessible, but their palaeoclimate is documented by the peripheral Rift and Afar Lakes⁶¹. The lake levels were highest, much above today, and they merged between 9,500 and 8,500 yr BP. The Ethiopian summer flood of the Nile was therefore much heavier. This seasonal variation accounts for the lamination of the sapropels: the white laminae are formed by coccoliths¹⁴ blooming with the flood, rich in dissolved ions of Ethiopian basalt origin.

The Nile Valley aggradation records these floods mostly from 12,000 to 11,000 yr BP, and from 8,000 to 7,000 yr BP in Sudan and Upper Egypt^{57,58,62}. There, aggradation indicates a "wild Nile with aberrant summer floods of a phenomenal volume"⁶². Moreover, higher local Sudanese (15° N) rainfall, which may have reached 550 mm yr⁻¹ (163 today)⁶³, between 8,000 and 7,000 yr BP (ref. 57), suggests decreased evaporation along the course of the Nile. Today, evaporation in the Sudanese swamps heavily reduces the Nile discharge.

The African and Indian monsoons, of equatorial westerlies origin, both controlled by the continental heat trough, displayed the same peak when the summer insolation was higher than today around 11,000 yr BP. At that time, strong monsoonal air flow and high rainfall in India is suggested by low sea-surface summer temperature in the Arabian Sea⁶⁴, due to strong upwelling induced by the southwesterly air flow, a mangrove much more extensive than today on the Indian coast near Bombay (E. Van Campo, personal communication), and river discharge higher than today in the Bay of Bengal⁶⁵. The Inter-tropical Convergence of the tropical easterlies, with its zenithal rains, was reactivated in East equatorial Africa.

From the eastern tropical Atlantic to the Bay of Bengal, along the inner tropics of the African continent and Indian Ocean, and in the other equatorial longitudes, as the summer insolation was highest, the late Glacial–early Holocene was a very wet period, the most humid of the past 20,000 yr. From around 12,500 yr BP, during the Bolling–Allerød interstadial, the

higher rainfall belt migrated progressively from the Equator polewards through the Preboreal and Boreal. The Atlantic was the wettest period in the 50–55° N lat. zone. It was set back by a drier episode which corresponds to the cold Younger Dryas, recorded in the Mediterranean sapropel by the disappearance of the brackish surface layer, thus interrupting the stagnation from 10,400 to 9,000 yr BP.

Conclusion

The Mediterranean two-layered upper sapropel in core KS52 is dated at 11,800–10,400 yr BP and 9,000–8,000 yr BP, the interruption resulting from the Younger Dryas cold/dry event. This is too late to be caused by the influx of Eurasian ice-sheet meltwater. Instead, oxygen and carbon isotope variations in planktonic foraminifera show that the light, low-salinity, organic and nutrient rich sea-surface layer which spread slightly before sapropel deposition and selectively increased the planktonic productivity, originated as rainfall. The pollen record around the eastern Mediterranean does not indicate much increased local rainfall at that time. Meanwhile, in equatorial Africa, very heavy rainfall began at 12,500 yr BP and culminated between 10,000 and 8,000 yr BP. We conclude that this equatorial deluge, channelled by the Nile River over 6,700 km is, as already suggested^{58,66}, the source of the surface brackish-water layer in the Mediterranean. This layer enhanced the vertical salinity and density gradient, and established stable stratification. Thus bottom stagnation was triggered in this hydrographically restricted deep basin, and sapropel formation resulted. The East Mediterranean behaved as an ectogenic meromictic lake as a stratified estuary and as a fjord during the stagnation events.

The establishment of these climate links have far-reaching geological implications.

(1) The Quaternary climatic chronology of Africa is recorded in the eastern Mediterranean subsurface. Sapropels are formed during all the periods of high summer insolation of the Northern Hemisphere displayed by the Milankovitch curve, which occur even during glacial isotopic stages 3, 4 and 6. The isotope stratigraphy supports these correlations.

(2) Widespread marine stagnation events occurred during certain short and well-defined periods of the Cretaceous. They deposited pelagic laminated sapropels and we suggest that they result from the same chain of events as this recent Mediterranean sapropel. In equatorial West Gondwana around the opening Atlantic, the thick fluvio-deltaic sediments of the Barreirinhas Basin suggest great river discharge in a seasonally very humid tropical climate, with a contrasting dry season enhancing evaporation. These Cretaceous sapropels may be important as possible petroleum generating environments.

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Polyoma virus capsid structure at 22.5 Å resolution

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X-ray diffraction data from polyoma capsid crystals were phased by refinement of low-resolution starting models to obtain a self-consistent structural solution. The unexpected result that the hexavalent morphological unit is a pentamer shows that specificity of bonding is not conserved among the protein subunits in the icosahedrally symmetric capsid.

X-RAY crystallographic studies on polyoma virus were begun to visualize the protein subunit packing in the icosahedral capsid and to explore the way in which the chromatin core of this tumorigenic virus is packaged inside its protein shell¹. Analysis of electron micrographs has established that the capsid consists of 12 five-coordinated and 60 six-coordinated morphological units² (capsomeres) arranged on a $T=7d$ icosahedral surface lattice³ (Fig. 1). It has been presumed, based on the principles formulated to account for the morphology and chemical composition of simple icosahedral virus particles⁴, that the capsids of the $T=7$ papova viruses should be constructed from 420 identical protein subunits quasi-equivalently bonded into 12 pentameric and 60 hexameric capsomeres. High-resolution X-ray structure determination of tomato bushy stunt virus⁵ and southern bean mosaic virus⁶ has established that quasi-equivalence is used to conserve essential bonding specificity among the 180 chemically identical subunits of these $T=3$ icosahedral capsids. Our 22.5-Å resolution electron density map of the polyoma capsid reveals unexpected substructure in the hexavalent capsomere that is inconsistent with the expectation of quasi-equivalent bonding of identical subunits. (The adjectives 'hexavalent' and 'pentavalent' are introduced to identify the six- and five-coordinated morphological units without prejudging the nature of their substructure.)

The starting point for our analysis of the polyoma capsid diffraction data was a model representing the coarse surface features seen in the three-dimensional image reconstruction from electron micrographs of negatively stained particles³. The capsomeres in this image reconstruction appear as hollow, stubby protrusions with no regular substructure, about 50 Å in diameter and separated from their nearest neighbours by 80–90 Å at a radius of 200 Å in the capsid. Pentavalent and

hexavalent morphological units appear to be about the same size, which would not be expected if they were composed of pentamers and hexamers of identical protein subunits.

Cells infected with polyoma virus and other papova viruses produce, in addition to intact virions and empty capsids, hollow tubular particles that appear to be polymorphic assemblies of the capsomeres⁷. There are two categories of tube: wide tubes,

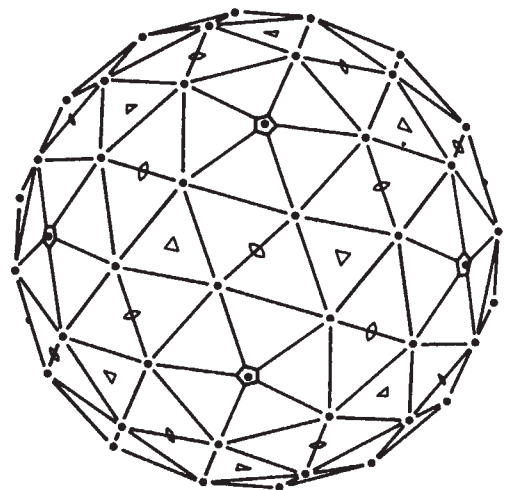


Fig. 1 $T=7d$ icosahedral surface lattice with five-, three- and two-fold axes marked. The drawing shows one side of the polyhedral surface consisting of 60 six-coordinated and 12 five-coordinated lattice points at the same radius. The location of the six-coordinated point is that determined for the hexavalent morphological unit in the polyoma capsid.

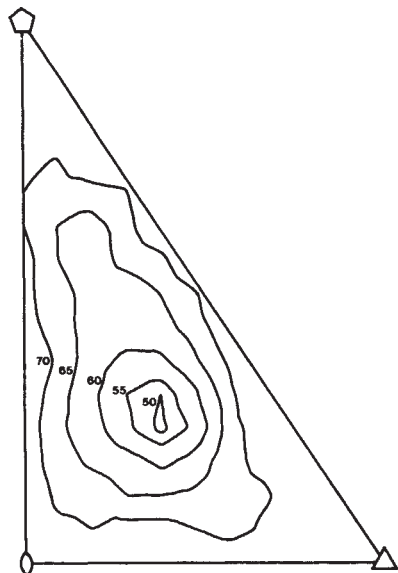


Fig. 2 Location of the hexavalent unit in the icosahedral surface lattice. The R factor comparing the calculated and measured diffraction pattern is plotted as a function of the position of the hexavalent unit in the icosahedral cell defined by the five-, three- and two-fold axes (see Fig. 1). The model giving the smallest R factor is shown in Fig. 3a.

about the diameter of the virion, which are built of capsomeres arranged in a hexagonal surface lattice, and narrow tubes, which Kislev and Klug⁸ showed to be constructed of pentameric capsomeres arranged in a lattice they called a 'pentagonal tessellation'. The capsomeres of the wide tubes were presumed to be hexamers but the chemical and structural relationships between the morphological units of the 'hexamer' and 'pentamer' tubes could not be established at that time. In the light of our results new questions are raised about the structure of these polymorphic assemblies of the capsid subunits.

Complete virions contain seven different proteins, four of which are cellular histones associated with the DNA, the other three (VP1, VP2 and VP3) being virus coded⁹. VP1 (molecular weight (MW) ~42,000) is the major capsid protein. The function of VP2 and VP3 (MW ~35,000 and ~23,000, respectively), which constitute less than 20% of the virion proteins, is not clear. Purified capsids have been prepared that contain only VP1¹⁰ and these particles have normal morphology. Thus, the pentavalent and hexavalent capsomeres seem to be built of identical VP1 subunits.

Crystallography

Polyoma virus capsids of the strain Sp-34 were isolated as previously described¹¹. The preparation used for growing crystals consisted predominantly of the major capsid protein, VP1, and only a small amount of VP2 and VP3 compared with the intact virion. Crystals, in the rhombic dodecahedral form¹², were grown by equilibrating a solution of polyoma virus capsids at 4 mg ml⁻¹ in 0.02 M Tris pH 9.5 and 0.17 M sodium sulphate against 0.55 M sodium sulphate by vapour diffusion. Crystals grew over a period of 1 month at room temperature. These crystals diffracted to a resolution of at least 8 Å. The icosahedral capsids, of packing diameter 495 Å, are located at the centre and corners of the b.c.c. lattice (space group I23, unit cell edge 572 Å) with particle two- and three-fold axes coincident with the crystallographic axes¹. Diffraction data were collected to 22.5 Å resolution by screened precession photography (crystal-to-film distance 150 mm) with an Elliott GX6 rotating anode X-ray generator using a 0.2-mm focal cup and two perpendicular-focusing mirrors¹³. A total of 11 zero level ($\mu = 2^\circ$) and 4 upper level ($\mu = 1.5^\circ$) photographs were collected, each a 48-h exposure. These gave 1,481 independent reflections between (1/150) and (1/22.5) Å spacing, representing 94% of the data within this resolution range. The individual films were digitized

on an Optronics P1000 densitometer at 50 μ m scanning raster and processed using a modified version of the Harvard Scan-11 system on a PDP-11/40 computer. The films scaled together with an R factor measuring the mean variation in intensity of symmetry-related reflections of 5.7%.

Models for phase refinement

Trial phases, calculated to 30 Å resolution from models based on information from electron microscopy³ and small-angle X-ray diffraction¹, were refined and extended to 22.5 Å resolution by imposing the constraints of non-crystallographic symmetry and solvent flattening. This approach is similar to that used to determine the structure of southern bean mosaic virus to 22.5 Å resolution¹⁴. Our starting model of the polyoma capsid, consisting of 72 morphological units arranged in a $T = 7d$ icosahedral surface lattice (Fig. 1), is a non-centrosymmetric structure. Consequently, calculated trial phases will introduce the non-centric component of the structure factors from the start. This is essential to the quality of the subsequent real-space phase refinement and extension.

Models consisting of 72 hollow cylindrical capsomeres on a spherical shell were formulated with 9 adjustable parameters defining the size and position of the structural elements. Phases from these models were calculated by Fourier transforming a density map generated in a lattice corresponding to the polyoma crystal. The model parameters were adjusted by comparing the calculated and observed structure factor amplitudes. The models which gave the best R factor fit were built from morphological units about 40 Å high and 80 Å in diameter with a 35-Å diameter axial hole, resting on a shell extending from a radius of 180–200 Å. The effect of moving the hexavalent morphological unit in this model is shown in Fig. 2, where the R factor is plotted as a function of position. The position of the minimum is close to the hexavalent capsomere location observed in Finch's image reconstruction from electron micrographs³. A projection of this 'best' model down a two-fold axis is shown in Fig. 3a. The R factor for this model to 30 Å resolution was 50% where R is defined as

$$R = \frac{\sum |F_o| - |F_c|}{\sum |F_o|} \times 100$$

where $|F_o|$ and $|F_c|$ are observed and calculated structure factor amplitudes. The R factor as a function of resolution is shown in Fig. 4a.

Particle envelope

Flattening the solvent regions of the electron density map places a powerful constraint on the low resolution phases and also provides the basis for phase extension. Thus, the envelope chosen to enclose the virus capsid is critical in the phase refinement and extension. The envelope used was a spherical shell extending from 165 Å to 260 Å truncated by planes perpendicular to the icosahedral three-fold axes 247 Å from the virus centre. These boundary planes bisect the line of contact between virus particles along the crystallographic three-fold axes. The three hexavalent units adjacent to the crystallographic three-fold axis make contact tip to tip with the related set on the neighbouring particle. As there is no discernible interdigitation of the capsomeres at this resolution, the boundary planes perpendicular to the three-fold axes define the volume associated with one virus particle.

Solvent density for both the interior and exterior of the capsid was taken as the average observed density of the shell extending from 160 to 165 Å. This approach was used because omitting unmeasured low-resolution $<(1/150)$ Å reflections in the initial calculations results in spurious electron density ripples inside the capsid. When the average density of the total solvent region defined by the particle envelope was used as suggested by Brice¹⁵, the refinement calculation converged more slowly. Nevertheless, both methods of determining the solvent density gave very similar final values.

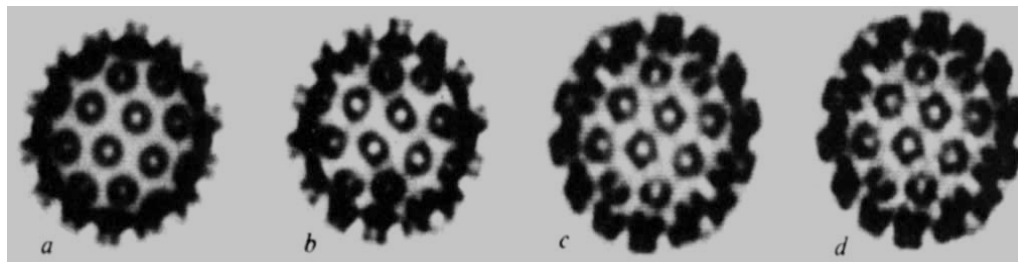


Fig. 3 Density maps of the top half of particles (diameter 495 Å) projected down a two-fold axis (orientation as in Fig. 1). The maps were scaled to have the same total electron density and were photographed directly from a 256 grey scale TV graphics display. *a*, Model map at 30 Å resolution; *b*, map computed using observed data and model phases to 30 Å resolution without refinement; *c*, 30 Å resolution refined map; *d*, refined map at 22.5 Å resolution calculated by phase extension from 30 Å. Note how the substructure in the morphological units develops as the refinement proceeds. Even at 30 Å resolution the hexavalent unit has a pentagonal appearance.

Refinement method

The calculated phases from the model structures were refined to 30 Å resolution against the observed structure factor amplitudes by imposing the constraints of solvent flattening and the five-fold non-crystallographic symmetry¹⁵⁻¹⁷. An electron density map was calculated to 30 Å resolution using the model phases and observed amplitudes, and averaged over the five non-crystallographic asymmetric units within the particle envelope. The observed amplitudes were weighted according to their fit to those calculated from the model according to the scheme

$$W = \exp \left(- \frac{|F_o| - |F_c|}{|F_o|} \right)$$

This simple weighting scheme gave faster convergence than that suggested by Sim¹⁸, but the final phase set was not sensitive to the weighting procedure used.

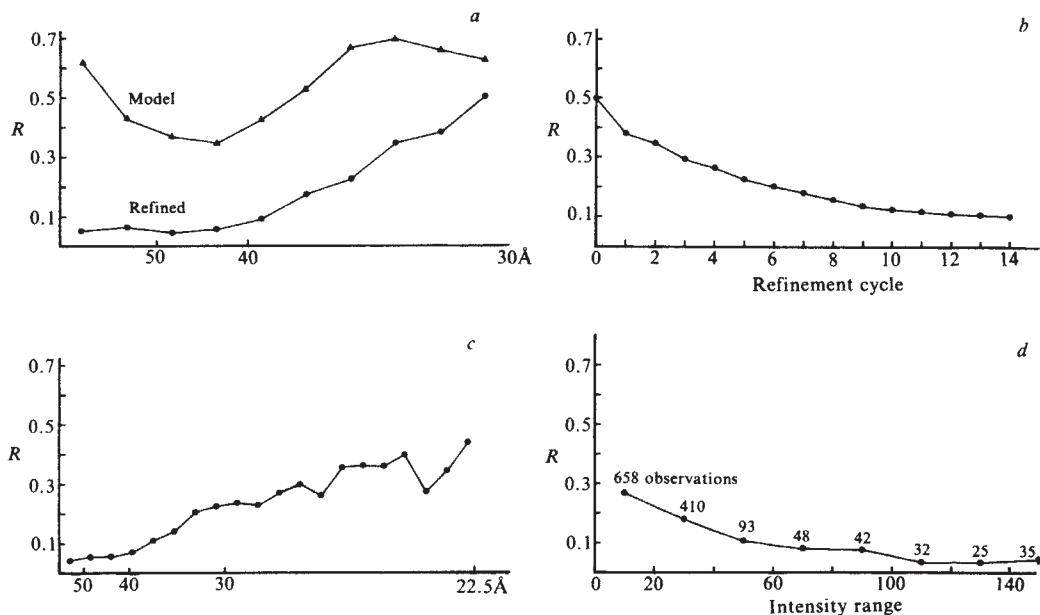
The averaged, solvent-flattened density map was Fourier transformed to calculate phases and amplitudes for the first cycle of refinement; the r.m.s. phase difference and *R* factor comparing initial and refined structure factors were evaluated; refined phases were combined with measured amplitudes to initiate the next cycle of refinement; these calculations were iterated until

the calculated phase difference and *R* factor did not change significantly from one cycle to the next.

There are two possible orientations for the icosahedral five-fold axis in the I23 unit cell. The orientation used was that indicated by the intensity spikes in the diffraction patterns¹. If the alternative orientation of the five-fold axis was chosen, the *R* factor between refined and observed structure factors never dropped below 50%. The standard deviation in the density between the five icosahedral units in the crystallographic asymmetric unit for the correct orientation was 1/7th of that calculated for the alternative.

Omission of the unmeasured data below (1/150) Å spacing systematically lowers the calculated amplitudes for the observed structure factors below (1/100) Å spacing. The amplitudes of the innermost observed reflections are the largest in the data set and dominate the scaling of the calculated to the observed structure factors. This effect was mitigated by including the calculated values for the unmeasured data after the fourth cycle with a weight of 0.9. Reduced weighting was applied to avoid divergence of these large terms in the iterative refinement. Various test calculations have established that the low order data can be accurately estimated from the higher resolution data. Without the eight calculated values for the missing low order data, the refinement converged to only 27% as compared with 10.5% with these data after 14 cycles of phase refinement.

Fig. 4 *R* factors comparing the agreement between observed and calculated structure factor amplitudes. *a*, As a function of resolution comparing initial model and refined map at 30 Å resolution; *b*, as a function of refinement cycle at 30 Å resolution; *c*, as a function of resolution for the phase extension from 30 to 22.5 Å resolution; *d*, as a function of intensity for the 22.5 Å refined map. For *a* and *c* the points are plotted so that the interval between points corresponds to equal volume increments in reciprocal space. The starting model (Fig. 3*a*) fits the observed data best in the 40–50 Å resolution range where there is a strong modulation in the transform determined by the surface lattice arrangement of the morphological units¹. After 14 cycles of refinement (*b*) there is a substantial improvement in the fit between the calculated and observed amplitudes at low resolution (*a*). The structure amplitudes phased by extension from 30 to 22.5 Å resolution represent over half of the total data to this resolution. The *R* factors for these higher resolution data (*c*) are uniformly satisfactory. The phase extension also leads to an improvement in the *R* factor for the data to 30 Å resolution by reducing series termination effects. Plotting the *R* factor of the 1,343 non-zero reflections as a function of intensity (*d*) shows that there is a correlation between the *R* factors and the accuracy of the data which are more precise for the stronger reflections.



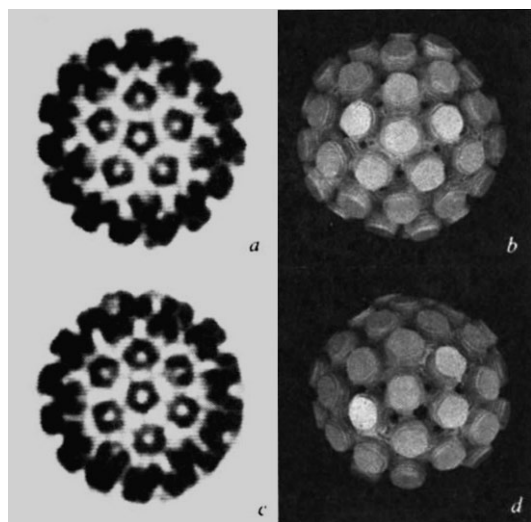


Fig. 5 Views of half the capsid (495 Å diameter) down the five-fold axis (*a, b*) and down the axis of the hexavalent unit (*c, d*). *a* and *c* are computer graphics projections of the electron density map at 22.5 Å resolution, *b* and *d* are photographs of a model of stacked sections of the map at 30 Å resolution with the external contour level set at 0.2 of the maximum density. The half-model was built symmetric about the five-fold axis (*b*), thus, when it is tilted to view down the hexavalent unit (*d*) the bottom portion of the image is incomplete.

Figures 3*a–c* and 4*b* illustrate the progress of the refinement in real and reciprocal space. Figure 4*b* shows the *R* factors as a function of refinement cycle. Figure 3*a* represents the starting model to 30 Å resolution, Fig. 3*b* the unrefined structure calculated from the observed amplitudes with model phases, and Fig. 3*c* the refined structure at 30 Å resolution.

Tests of refinement method

There is no established criterion for determining the correctness of the electron density map described subsequently. Because the map shows unexpected features, considerable effort was devoted to demonstrate the uniqueness of the refinement. Phases from any model consisting of 72 bumps distributed on the surface of a spherical shell, in a manner consistent with the image from electron microscopy³, refined to the same solution. Indeed, a starting model which placed 60 hemispheres close to the location of the hexavalent units on a spherical shell with no density protruding on the five-fold axes (initial *R* factor = 65%) refined to the same phase set as the more realistic models, although it took 18 cycles of refinement. Furthermore, phases from elaborate models with hexavalent morphological units containing either five- or six-fold substructure refined to the same final structure.

The reliability of the refinement method was tested by using amplitudes calculated from models in place of observed data. Because the model structure factor calculations give both the amplitude and the phase, it is possible to judge absolutely how well another set of trial phases converges to the correct values. In the test calculations, amplitudes from various *T* = 7 capsid models were combined with phases from models with different morphology; refinement of the phases always converged to the phases of the model from which the amplitudes were taken. For example, refining initial phases from an all-pentamer *T* = 7 model against the amplitudes from a 60 hexamer + 12 pentamer model led back to the phases of the model built with hexamers. Thus, our refinement procedure recovers the correct structure corresponding to the amplitudes of the data even when trial starting phases were chosen to attempt to bias the calculation to produce an incorrect result.

Phase extension

The polyoma diffraction data between 30 and 22.5 Å resolution were phased by extension from the refined 30 Å phases. This

was necessary because the model phases are only reliable to ~30 Å resolution. Attempts to refine the data directly to 22.5 Å resolution using trial model phases did not converge satisfactorily.

Phase extension is possible because imposition of the envelope on the electron density generates phase information for reflections just beyond the resolution of those used to calculate the map. The characteristics of phase extension were tested using model structure factors and phases in place of the real polyoma diffraction data. Starting from 30 Å resolution, using calculated amplitudes and phases as test data, it was possible to extend and refine the phases to 22.5 Å resolution. The r.m.s. phase difference between the correct and the refined extended phases were generally less than 30°.

The resolution of the actual data refinement was extended from 30 Å to 22.5 Å over 18 cycles. Predicted phases for structure factors within one reciprocal lattice distance (1/572) Å of the previous resolution were included in alternate cycles. The final *R* factor between observed and calculated structure factor

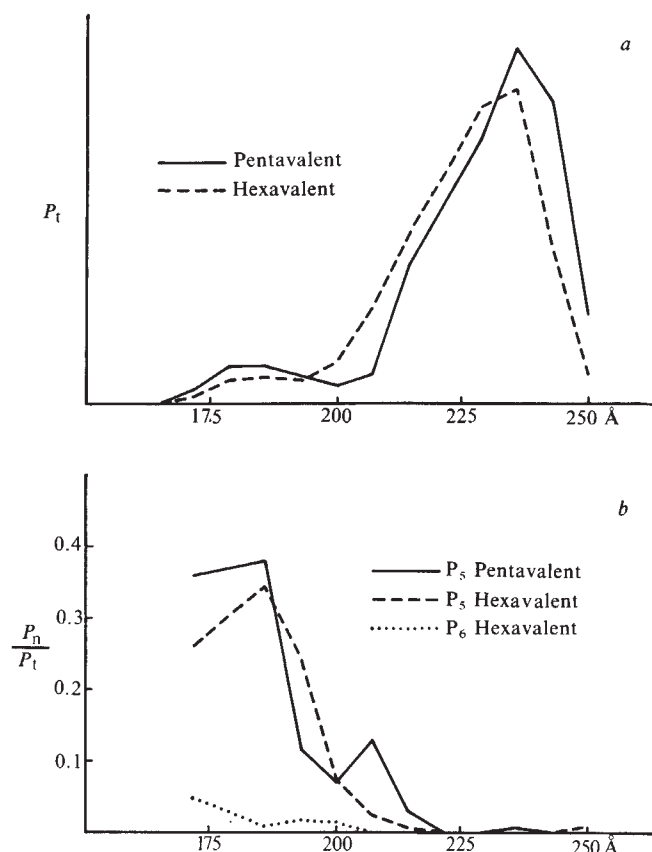
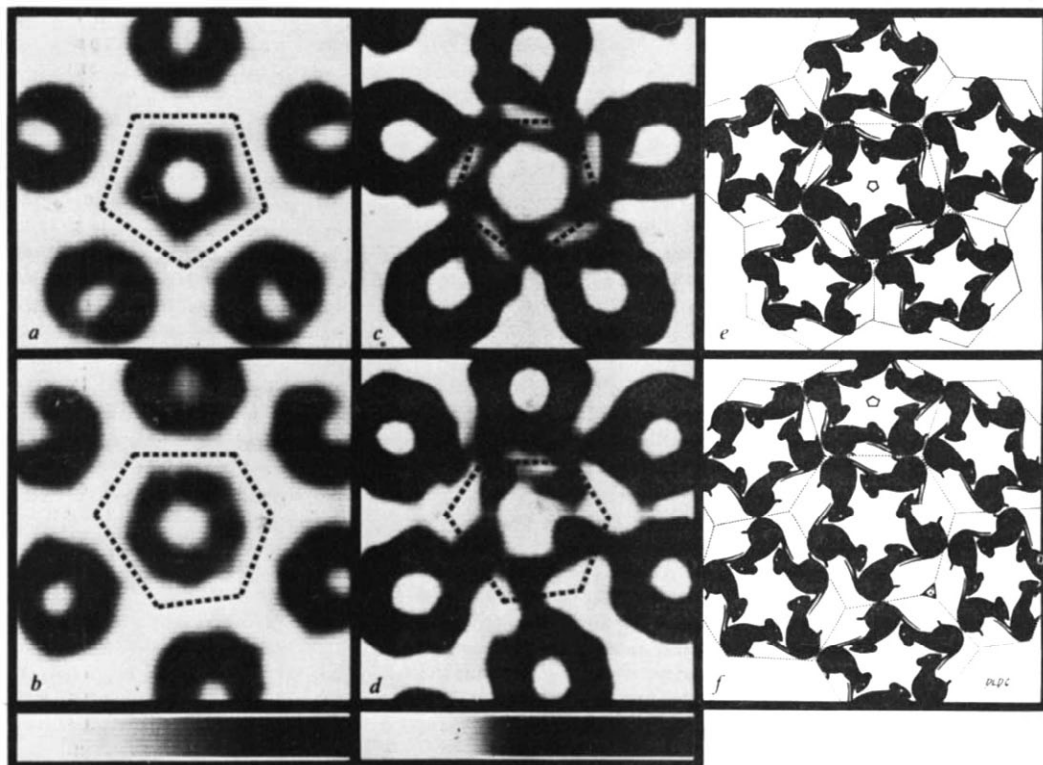


Fig. 6 Rotational power spectra of the pentavalent and hexavalent units. *a*, P_t is the sum of all cylindrical harmonics in sections of the map through the capsomeres normal to the five-fold axis and the 'best' axis of the hexavalent units, plotted as a function of the distance of the section from the centre of the particle. *b*, Relative strength of the five-fold harmonic of the pentavalent unit and the five- and six-fold harmonic of the hexavalent unit. P_t is proportional to the integrated density in each section. At small radii in the capsid a cylindrical boundary of 93 Å diameter was chosen for calculating the power spectrum of the capsomeres. These cylinders touch at a radius of 170 Å. The integrated density of the inner portions of the capsomere may be underestimated by the methods of calculation. The correspondence in the rotational power spectra distribution of the pentavalent and hexavalent units indicates a close similarity in their structures and a small difference in their radial positions. The relative strength of the order 5 harmonic for the outer portion of both the pentavalent and hexavalent capsomere is reduced because the cylindrically symmetric component, which is the dominant term in P_t , is very strong for this part of the structure. The order 5 harmonic for the hexavalent capsomere is always much stronger than that of order 6 (or any other non-zero harmonic).

Fig. 7 Sections of the electron density map showing substructure of the capsomeres (*a-d*) and drawings on a polyhedral surface (*e, f*) illustrating an inferred packing relation of structure units. Upper row: views down five-fold axis and lower row: views down axis of hexavalent capsomere. Width of sections displayed 215 Å. Orientation as in Fig. 5. Grey scales at the bottom show the relation between darkness in the images and map density that increases uniformly from left to right of the scales. *a, b*, Maps of section planes 215 Å above centre of capsid. At this level and above there is little detectable contact between neighbouring capsomeres. *c, d*, Maps (displayed with enhanced contrast) of section planes 195 Å above centre. At this level and below five basal parts of the capsomeres splay out and contact basal parts of neighbouring capsomeres. Section planes are perpendicular to the axis of the central capsomere and slice through the neighbouring capsomeres obliquely. In the obliquely sectioned parts the substructure is obscured. Intersections of adjacent planes equidistant from the centre of the capsid that are normal to the axes of the pentavalent and hexavalent capsomeres define 12 equal regular pentagons and 60 equal irregular hexagons. The polygons delineating the domain of the central capsomeres are marked by dotted lines on the density maps. The drawings *e* and *f* (constructed on the polyhedral surface made up of the pentagonal and hexagonal facets) illustrate that bonding specificity among identical structure units (represented by graphically cloned mice) cannot be conserved in all the contacts between capsomeres. Arranging structure units in the hexavalent pentamer (centre of *f*) with quasi-five-fold symmetry resembling the pentagonally symmetric pentamer (centre of *e*) leads to three types of contacts between structure units of neighbouring pentamers: (1) the two structure units at the top of the central pentamer in *f* each contact two neighbours (one from the pentavalent and one from another hexavalent pentamer) related by a quasi-three-fold axis; (2) the two structure units below and right of centre in *f* make quasi-two-fold contacts with neighbours related by an icosahedral three-fold axis; (3) the one structure unit left of centre in *f* makes an intimate dimer contact with a unit equivalently related by an icosahedral two-fold axis. Thus, the six structure units in the icosahedral asymmetric unit can be put into three categories according to their bonding relations: three in the quasi-trimer, two in the quasi-dimer and one in half the strict dimer.



amplitudes was 14.2%. Figure 3*d* shows a projection down a two-fold axis of half a virus particle excised from the refined map at 22.5 Å resolution and an analysis of the *R* factor as a function of resolution and intensity at 22.5 Å resolution is shown in Fig. 4*c, d*.

Electron density map

Figure 5, comparing views of half a virus particle down the icosahedral five-fold axis with that down the axis of a hexavalent morphological unit, reveals a striking similarity between the penta- and hexavalent morphological units. Indeed, the hexavalent capsomere shows a distinctive five-fold character, not six-fold as would be expected from a *T* = 7 icosahedral surface lattice consisting of 60 *T* quasi-equivalent structure units (Fig. 1).

Power spectra of the cylindrical harmonics in sections through the penta- and hexavalent units are shown in Fig. 6. This harmonic analysis demonstrates the structural similarity between the two types of capsomeres as a function of their radial coordinates, in particular, the dominant five-fold substructure of the hexavalent unit. Icosahedral symmetry requires the 12 identical pentavalent units to have five-fold symmetry and the 60 hexavalent units to be identical; but there are no constraints on the size, shape or symmetry of the hexavalent unit. The similarity of the pentavalent and hexavalent capsomeres seen in the electron density map cannot be an artefact of the imposition of the non-crystallographic symmetry in the phase refinement.

Thus, our results indicate that all 72 capsomeres are pentameres of identical or very similar subunits.

The electron density map may be divided into two regions: the protruding pentagonal caps and the basal parts connecting capsomeres. The contacts between capsomeres extend inward from ~210 Å radius of the capsid. The protruding caps are ~85 Å in diameter and extend out ~40 Å. The central hole in the capsomere has a diameter of 40 Å at a radius of 210 Å and tapers shut 15 Å from the surface, leaving a dimple on the top of the cap. The basal parts of the capsomeres extend in ~40 Å.

The similarity of the pentavalent and hexavalent morphological units seen in the electron density map, together with the chemical evidence for one kind of protein subunit, indicates that the capsid is built of 360 VP1 molecules. The radial extent of the capsid measured from the electron density map implies a protein subunit ~80 Å long. The dimensions of the wall of the pentagonal cap indicate a subunit diameter in the range 30–40 Å. An ellipsoidal subunit with these dimensions has a volume compatible with that expected for a 42,000-MW protein.

Molecular weight

All the available MW measurements are consistent with a capsid built of 360 VP1 subunits and inconsistent with a 420-subunit structure. The MW of the empty capsid, determined by sedimentation equilibrium¹⁰, is $15 \pm 1 \times 10^6$, which agrees with the total weight of VP1 in the intact virion estimated from its MW and composition. Furthermore, the MW of 221,000 measured

for isolated capsomeres¹⁹ corresponds to a 15.9×10^6 -MW capsid built of 72 identical capsomeres, in accord with the direct measurement. The calculated MW of VP1 for a $15 \pm 1 \times 10^6$ -MW capsid consisting of 420 subunits would be $35,700 \pm 7\%$, or $41,700 \pm 7\%$ for 360 copies. The calculated MW of VP1 from the DNA sequence is 42,834 (ref. 20) and 42,458 (ref. 21) for two strains of polyoma virus. These sequence values are upper limits because the protein in the capsid may be smaller due to possible post-transcriptional or post-translational processing. Estimates for the MW of VP1 from SDS-gel electrophoresis range from 42,000 to 50,000 but are not very reliable because of dependence on gel concentration¹⁰. Measurement by gel exclusion chromatography of VP1 dissociated with guanidine hydrochloride gives a MW of 41,400 (ref. 10). Although there is no definitive MW measurement for the VP1 subunit in the capsid, all measured values are greater than or close to that expected for 360 subunits, and the smallest measured values are at least 15% larger than expected for 420 subunits.

Protein-subunit interactions

The electron density map provides information about subunit interactions even though the protein subunits themselves are not sharply resolved. Figure 7a and b are sections through the hexavalent and pentavalent capsomeres above the level at which they make contact with each other. It is evident that the projecting portions of the two kinds of capsomeres have very similar pentagonal substructure, indicating that the bonding specificity of the projecting parts of the structure units is conserved. This is analogous to the situation in the tomato bushy stunt virus structure⁵ where bonding specificity is conserved within the two types of protruding dimer domains.

Where the polyoma capsomeres contact each other there are significant differences in the bonding as the five subunits of the hexavalent pentamer cannot interact equivalently with subunits of the six neighbouring pentamers. This is seen in Fig. 7c and d, which are sections at the level of contact between capsomeres. At this resolution, subunit boundaries cannot be distinguished; however, it is possible to suggest the types of interaction which would account for the observed density.

Our results show there are six structure units in the icosahedral asymmetric unit, five from the hexavalent and one from the pentavalent pentamer. Each of these subunits has a symmetrically distinct environment in the capsid. However, these six units can be put into three different classes according to their bonding relations inferred from the electron density map. These relations of the six structure units in the icosahedral asymmetric unit are schematically illustrated in Fig. 7e and f: three units are connected in one quasi-trimer, two in one quasi-dimer and one in half a strict dimer. The use of the three types of bonds can account for the assembly of the 72 pentamers in the capsid; but we do not know how the bonding specificity of the structure units is switched to control the assembly process.

Conclusion

The electron density map shows a hexavalent capsomere that closely resembles the regular pentameric capsomere in size, shape and substructure. Because the hexavalent capsomere was expected to be a hexamer, the reliability of the refinement

method used to solve the structure has been critically examined. In contrast to the isomorphous replacement method in which the phase determination is constrained by measurement of heavy atom positions, and the Fourier refinement method at atomic resolution which is constrained by detailed stereochemical information, our refinement method, starting with trial phases from models representing the coarse particle morphology, is only constrained by the icosahedral symmetry and overall dimensions of the virus capsid. Our tests of the refinement method, applied to Fourier transforms calculated from capsid models to the same resolution as the experimental data, show that the constraints of the particle symmetry and overall size do lead to correct solutions starting with trial phases from any model with approximately related capsomere morphology. We believe, therefore, that the similarity of the hexavalent and pentavalent capsomeres seen in the electron density map is an intrinsic feature of the virus capsid structure rather than some unexplained artefact of the refinement procedure. As chemical analysis indicates that the capsid is built of VP1, it seems that both the hexavalent and pentavalent capsomeres are pentamers of VP1.

The similarities between polyoma virus, SV40 and other members of the papova family suggest that these may all have capsids built of pentameric capsomeres. Polymorphic tube structures are associated with all the papovaviruses. The common occurrence of pentamer tubes is understandable if all the capsomeres are pentameric. We suspect that the hexamer tubes are also made of pentamers. Re-examination of the published electron micrographs^{7,8} indicates that this may indeed be the case. We are starting image analysis of electron micrographs to try to understand how the polymorphism of these structures arises.

Quasi-equivalence was conceived to explain why icosahedral symmetry should be selected for the design of closed containers built of a large number of identical structure units with conserved bonding specificity⁴. However, as bonding specificity is not conserved in the polyoma capsid, the reason for its icosahedral symmetry is no longer obvious. Why has an icosahedral capsid design of 72 pentamers been selected for polyoma and presumably other papova viruses? How is the bonding specificity of the structure unit switched to fit into the symmetrically distinct environments? Answers to these questions may be found by solving the capsid structure at higher resolution and by studying the structure of assembly intermediates.

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Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells

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*Polyadenylated RNAs of certain human tumour cell lines are shown to contain transcripts related to the cell-derived transforming *onc* genes of molecularly cloned primate, murine or avian transforming retrovirus genomes. Thus, analogues of retroviral transforming genes are both present and frequently expressed in human neoplastic cells.*

THE study of RNA tumour viruses, designated transforming retroviruses, has provided a potentially important approach to elucidating mechanisms involved in human malignancy. These agents generally cause sarcomas and haematopoietic malignancies, but in some cases also induce carcinomas (for reviews see refs 1–4). There is convincing evidence that these viruses have arisen by recombination of replication-competent type C RNA viruses with evolutionarily well conserved cellular genes. Moreover, these latter sequences, termed *onc* genes, have been found to be required for the induction and/or maintenance of viral transformation^{5–11}. Certain independent sarcoma virus isolates of the same^{12–15} and even of different species^{16,17} have been found to contain the same or closely related *onc* genes. These findings suggest that only a limited set of distinct cellular genes with transforming potential exists.

Molecular cloning techniques have facilitated the isolation and amplification of transforming retrovirus genomes and their cell-derived *onc* genes for detailed biochemical analysis. To investigate the possible role of *onc*-related genes in human neoplasia, we have analysed whether cellular DNA analogues of these genes are present and actively transcribed in human cancer cells.

Transforming retroviral *onc* genes as probes for detecting related transcripts in human cells

Our study required that the transforming retroviruses should meet certain criteria: (1) They should be capable of inducing a wide range of malignancies. (2) The genes used should be available as well defined genetic segments of molecularly cloned viruses. (3) Such gene segments should possess sufficient homology with human DNA to detect related information at low copy number with reasonably high signal intensity. Molecularly cloned transforming retroviruses meeting these criteria included simian sarcoma virus (SSV)^{18–20}, BALB murine sarcoma virus (MSV)²¹, Abelson murine leukaemia virus (MuLV)²² and MC-29 virus²³. SSV and BALB MSV induce sarcomas in susceptible hosts^{24,25}, whereas Abelson MuLV causes lymphoid tumours²⁶. *In vitro* and *in vivo* studies have indicated that other cell types can be targets for transformation by both Abelson MuLV and BALB MSV (refs 27, 28 and J. Hoelzer Pierce and S.A.A., unpublished observations). MC-29 has the widest known range of target cells and has been reported to induce sarcomas, haematopoietic tumours and carcinomas².

Infectious DNA clones of each virus were obtained by recombinant DNA techniques and their cell-derived *onc* genes defined by restriction endonuclease and heteroduplex mapping techniques^{18–23}. Figure 1 shows the physical maps of the molecular clones and indicates the region of each viral genome used

to prepare DNA probes. According to recent convention, the cell-derived *onc* sequences of SSV are designated *sis*, while those of Abelson MuLV, BALB MSV and MC-29 are termed *abl*, *bas* and *myc*, respectively²⁹.

Nick-translated DNA probes of each viral *onc* gene were first analysed for their ability to detect homologous sequences in human DNA. Normal human cellular DNA was cleaved with *Eco*RI, a restriction enzyme that did not cut within any of the viral *onc* genes (Fig. 1). The DNA was then subjected to agarose gel electrophoresis and Southern Blotting analysis³⁰. As shown in Fig. 2, each viral *onc* DNA probe detected one or at most a few DNA fragments. The signal intensity was highest with the SSV *sis* DNA probe, probably reflecting its greater homology with human DNA than that of the other viral *onc* genes analysed. The DNA fragments hybridized by each viral *onc* gene had different molecular weights, indicating that each detected a different homologue within human DNA. Lack of relatedness among the viral *onc* genes was confirmed by experiments in which each nick-translated fragment was hybridized to Southern blots containing the four unlabelled viral *onc* DNAs. Even in relaxed hybridization conditions³¹, there was no detectable relatedness among the *onc* genes of SSV, BALB MSV, Abelson MuLV or MC-29 (data not shown).

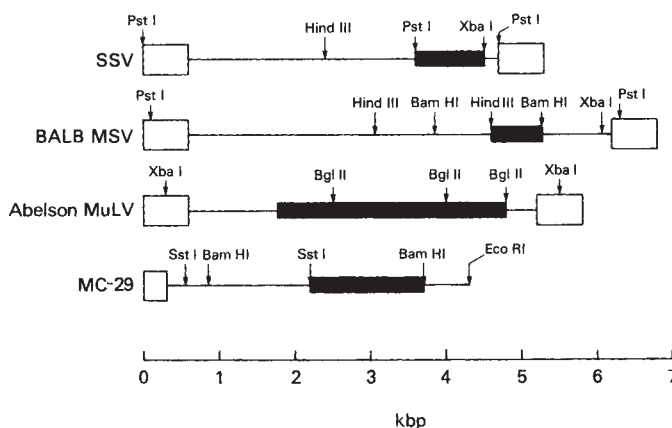


Fig. 1 Restriction maps of transforming retroviral genomes used to detect *onc*-related sequences in human cells. The *onc* genes of each retrovirus are indicated by the solid boxes. The white boxes represent the long terminal direct repeats (LTRs). Pertinent restriction sites are indicated. The integrated biologically active form of each viral genome was cloned in a λ phage vector^{18–23}. Host cellular flanking sequences are not shown. DNA probes were derived from subgenomic fragments of each viral *onc* gene as follows: SSV, *Pst*I–*Xba*I fragment; BALB MSV, *Hind*III–*Bam*HI fragment; Abelson MuLV, 5' *Bgl*II–*Bgl*II fragment; MC-29, *Sst*I–*Bam*HI fragment. With the exception of SSV, each subgenomic DNA fragment was cloned in pBR322.

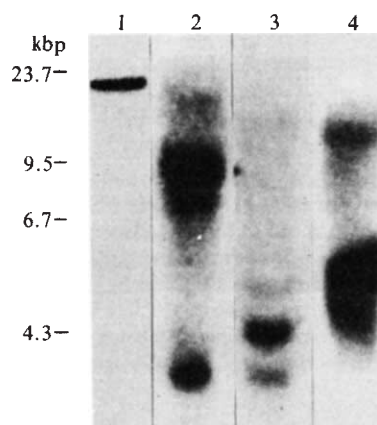


Fig. 2 Detection of human DNA sequences related to transforming retroviral *onc* genes. High-molecular weight human placental DNA was digested with *Eco*RI and electrophoresed on 0.8% agarose gels. Southern blots were prepared and incubated for 24 h with 2×10^6 c.p.m. ml^{-1} nick-translated (2×10^6 c.p.m. per μg) viral *onc* probes prepared from cloned DNAs of SSV (lane 1), BALB MSV (lane 2), Abelson MuLV (lane 3) and MC-29 (lane 4). Hybridization with the SSV *sis* probe was performed at 50 °C in 50% formamide and 5×SSC followed by washing at 50 °C with 6×SSC as described previously¹⁷. The other probes were hybridized at 65 °C in 6×SSC and washed at 45 °C in 0.1×SSC–0.1% SDS according to Hieter *et al.*⁴⁹. The blot in lane 1 was exposed for 16 h, and those in lanes 2–4 were exposed for 48 h using Kodak XAR-5 film and Dupont Cronex Lightning Plus screens. *Hind*III-generated fragments of bacteriophage λ DNA were used as molecular weight standards: kbp, kilobase pairs.

In our search for transcripts related to viral *onc* genes in human tumours, we selected tumour cell lines representing a diverse spectrum of malignancies, including carcinomas, sarcomas, melanomas and glioblastomas. The establishment and biological characterization of some of these cell lines have been reported previously (Table 1). Each cell line was malignant in athymic nude mice and reproduced the histopathology of the original tumour (refs 32, 33 and S.A.A. and N. W. Ellmore, unpublished observations). These established lines enabled us to conduct a systematic analysis of viral *onc* gene expression in human malignancies.

The approach used to detect *onc*-related transcripts in human tumour cells involved isolation of poly(A)-containing RNAs and analysis by agarose gel electrophoresis and Northern blotting techniques^{34–36}. Selection of poly(A)-containing RNAs provided the most sensitive method of detecting transcripts with properties of most known mRNAs, the sensitivity of detection of viral RNA in a typical retrovirus-transformed nonproducer cell being enhanced 30–40 fold by this procedure. This enhancement was consistent with the enrichment expected from the known fraction of poly(A)-containing RNA in total cellular RNA³⁷ and the percentage of poly(A)-containing RNA recovered by oligo(dT) selection³⁴. Human cells were routinely tested for the presence of viral *onc*-related transcripts both as

total and poly(A)-selected RNAs. In no case did we detect transcripts in total RNA that were not present in the poly(A) fraction. All cell lines were analysed for transcripts related to each viral *onc* gene. In some experiments, blots were sequentially hybridized with different *onc* probes as an internal control of the level of expression of the different *onc*-related transcripts.

SSV *sis* gene expression specific for certain human tumour cells

Nick-translated SSV *sis* DNA detected a single 4.2-kilobase (kb) transcript in 8 of 23 human tumour cell lines analysed, including 5 of 6 sarcoma cell lines and 3 of 5 glioblastoma lines. As shown in Fig. 3, the signal intensity of this transcript varied, the highest level being detected in a human glioblastoma line, A172 (Fig. 3, lane 1). The transcript was present at somewhat lower level in each of the positive sarcoma cell lines, as indicated for representative osteosarcoma and fibrosarcoma lines (Fig. 3, lanes 2 and 3, respectively). A second RNA species of lower molecular weight and much lower signal intensity was occasionally detected in some of the cell lines showing the 4.2-kb transcript. SSV *sis* gene expression was specific for these tumours as RNA isolated from normal human embryonic fibroblasts did not detectably hybridize (Fig. 3, lane 4). Furthermore, the SSV *sis*-related transcript was not observed in poly(A)-selected RNAs of any carcinoma, melanoma or other tumour cell line analysed. Note that SSV nonproducer cell RNA (Fig. 3, lane 5) exhibited genomic and one subgenomic RNA species containing *sis* sequences, neither of which corresponded in size to that of the 4.2-kb *sis*-related transcript in human sarcomas or glioblastoma cells.

A single MC-29 *myc*-related transcript in human tumour cells of different origins

The MC-29 *myc* probe, like SSV *sis*, detected a single transcript in human tumour cells. As shown in Fig. 4, this 2.7-kb transcript was observed in all cell lines examined, including normal human fibroblasts. Although the Northern blotting technique is not highly quantitative, there seemed to be a striking increase in the levels of *myc*-related RNA in some tumour cell lines analysed. In repeated experiments with the same RNAs or with RNAs independently prepared from the same cell lines, we reproducibly observed a 5–10-fold higher signal intensity of this transcript in lines derived from a sarcoma (A1383) and two carcinomas (A2780 and A549) as well as in the NC37 B-lymphoid line (Fig. 5). NC37 cells were initially established from Epstein-Barr virus (EBV)-positive lymphoid cells of a normal human³⁸. However, NC37 cells also possess an abnormal karyotype and are tumorigenic in nude mice (unpublished observations), indicating their malignant phenotype. Notably, there was no correlation between those cell lines demonstrating high levels of the 2.7-kb MC29 *myc*-related RNA and those containing the 4.2-kb SSV *sis*-related transcript.

Table 1 Human tumour cell lines studied

Origin	Cell line	Ref.	Origin	Cell line	Ref.	Origin	Cell line	Ref.
Sarcoma			Carcinoma			Glioblastoma		
rhabdomyo-osteogenic	A673	32	skin	A388	32		A172	32
	H0S	46		A431	32		A1235	–
	A2394	–	lung	A549	32		A1207	–
fibro-	8387	47	GI	A1617	–		A1690	–
	A1383	–	renal	A498	32		A2781	–
synovial	A2095	–	ovarian	A1847	–			
			ovarian	A2780	–			
Melanoma			Teratoma			Other		
	A101D	32		Tera I	33	B lymphoid	NC37	38
	A375	32				normal fibroblast	M413	48
	A1306	–						

In the columns of references, – indicates unpublished work.

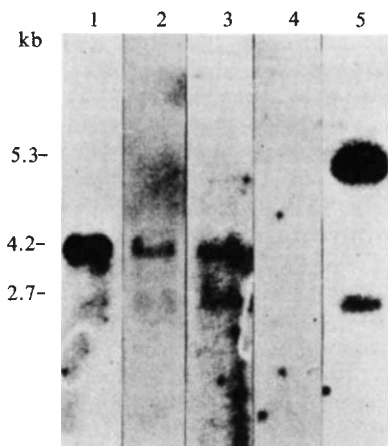


Fig. 3 SSV *sis*-related transcripts in human tumour cell lines. Total cellular RNA was isolated by a modification of the guanidine hydrochloride extraction method⁵⁰ as described elsewhere⁵¹. Poly(A)-containing RNA was selected by one cycle of chromatography on oligo(dT)-cellulose columns³⁴. Poly(A)-containing RNAs (5 µg per lane) were denatured in 13 mM methyl mercury hydroxide and size fractionated on 1% agarose gels containing 5 mM methyl mercury³⁵ and transferred to nitrocellulose filters as described previously³⁶. RNAs containing SSV *sis*-related sequences were identified by hybridization with 2.5×10^6 c.p.m. μl^{-1} nick-translated (2×10^8 c.p.m. per µg) *Pst*I-*Xba*I DNA fragment of molecularly cloned integrated SSV DNA¹⁸⁻²⁰. Hybridization with the SSV *sis* probe was performed in 20% formamide, $5 \times \text{SSC}$ at 37 °C for 36–48 h, followed by washing at 42 °C in $0.1 \times \text{SSC}$, 0.1% SDS as described previously^{36,49}. SSV *sis*-related transcripts were visualized by exposing the blots to X-ray films for 5–7 days with intensifying screens. Poly(A)-selected RNAs included those from: A172 glioblastoma cells (lane 1); HOS and 8387 human sarcoma cells (lanes 2 and 3); M413 normal human fibroblasts (lane 4); SSV nonproducer NRK cells (lane 5).

Multiple transcripts related to mouse *onc* genes in human tumour cells

onc gene probes of two mouse-derived transforming viruses, BALB MSV and Abelson MuLV, each detected related transcripts in all human tumour cell lines analysed. Figure 6 shows the results with representative tumour cell lines. The pattern of BALB MSV *bas*-related transcripts was complex (Fig. 6a), showing three distinct bands of 7.0, 5.9 and 1.8 kb, in many tumours. Some tumour cell lines seemed to contain a much lower level of the 1.8-kb transcript whereas in other cells,

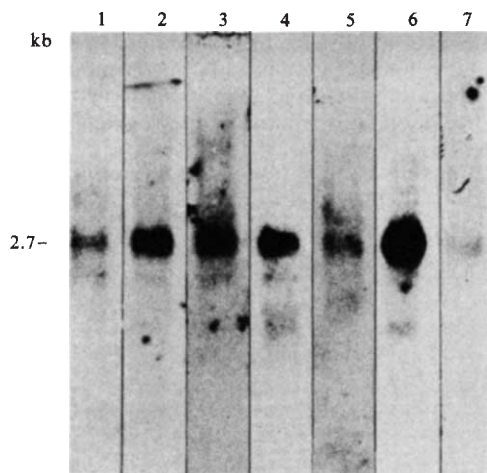


Fig. 4 MC-29 *myc*-related transcripts in human tumour cells. Poly(A)-selected RNAs were prepared and analysed as described in Fig. 3 legend. MC-29 *myc*-related transcripts were identified by hybridization with 2.5×10^6 c.p.m. ml^{-1} nick-translated *Sst*I-*Bam*HI DNA fragment of molecularly cloned integrated MC-29 DNA²³. Poly(A)-selected RNAs include those from: A2394 and A2095 sarcoma cell lines (lanes 1 and 2); A2780 and A549 carcinoma cell lines (lanes 3 and 4); A375 melanoma cell line (lane 5); NC37 B-lymphoid cell line (lane 6); M413 normal human fibroblasts (lane 7).

including normal human fibroblasts, this RNA species was not detected.

An even more complex pattern of transcripts was observed with the Abelson MuLV *abl* probe. At least five RNA species were detected in most of the tumour cell lines analysed (Fig. 6b), while normal fibroblasts demonstrated only the two largest *abl*-related transcripts. The widespread presence in tumours and even normal fibroblasts of multiple transcripts related to the *onc* genes of BALB MSV and Abelson MuLV suggests that expression of related human genes may be associated with an important cellular function(s). Whether the multiple RNAs observed with these probes reflect RNA processing or transcription of more than one member of a family of related *onc* genes remains to be determined.

Implications of retroviral *onc* gene expression in human cells

The present report demonstrates that sequences related to the *onc* genes of different transforming retroviruses are not only present in human DNA but can also be actively transcribed. Retroviral *onc* genes have been transduced by type C RNA

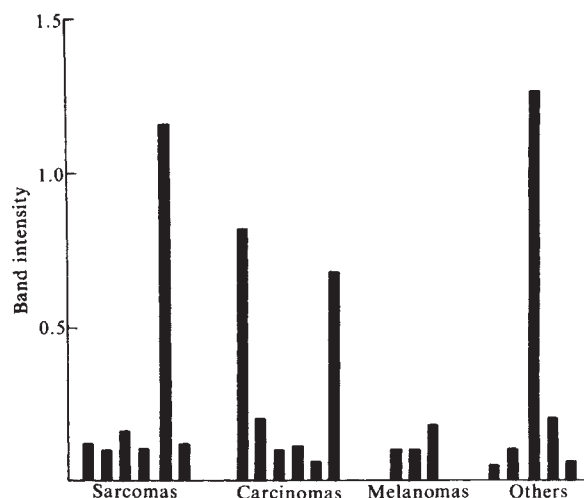


Fig. 5 Quantitative analysis of *myc*-related transcripts in human tumour cell lines. Autoradiographs obtained by exposure to X-ray films of RNA blots of different human cell lines hybridized to ^{32}P -labelled *myc* probe were scanned for absorbance at 340 nm in an ISCO absorbance monitor, model UA-5. Band intensities were obtained after subtraction of film background. Sarcoma cell lines were: 8387, HOS, A2095, A2394, A1383 and A673. Carcinoma cell lines: A2780, A1617, A498, A1847, A431 and A549. Melanoma cell lines: A1306, A101D and A375. Other cell lines were, respectively: glioblastoma cell lines, A172 and A1235, lymphoid B-cell line, NC37, a teratoma cell line, Tera I, and normal human fibroblasts, M413.

viruses from the genomes of species as diverse as primates, mice and chickens (for reviews see refs 1–4), and their presence in the DNAs of diverse vertebrate species, including man, indicates that such genes are highly conserved. Our findings that these genes are actively transcribed in human normal and/or neoplastic cells as polyadenylated RNA species provides additional evidence for the functional nature of these well conserved genetic elements.

It is clearly important to determine whether viral *onc* genes are critical to the transformation process in humans. It has recently been shown that genetic manipulation of a cloned normal mouse cell DNA analogue of one transforming retroviral *onc* gene so as to cause it to be transcriptionally activated, confers on it the ability to transform mouse cells^{9,39}. Moreover, other studies have indicated that leukaemia viruses may induce tumours by a mechanism involving 'downstream' promotion and transcriptional activation of cellular gene(s)⁴⁰⁻⁴². The markedly elevated levels of single transcripts related to either SSV or MC29 *onc* genes in certain human tumour cells are consistent with the transcriptional derepression of these genes. Thus, if

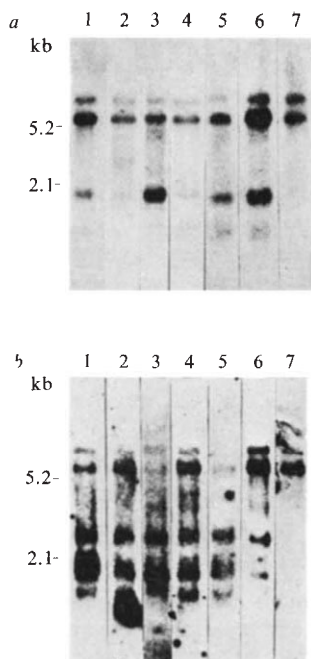


Fig. 6 BALB MSV and Abelson MuLV *onc* gene-related transcripts in human tumour cells. Poly(A)-selected RNAs were prepared and analysed as described in Fig. 3 legend. *a*, BALB MSV *bas*-related transcripts were identified by hybridization with 2.5×10^6 c.p.m. ml^{-1} nick-translated *Hind*III-*Bam*HI DNA fragment of molecularly cloned integrated BALB MSV DNA²¹. Poly(A)-selected RNAs include those from: A2095 and HOS sarcoma cell lines (lanes 1 and 2); A549 and A2780 carcinoma cell lines (lanes 3 and 4); A1306 melanoma cell line (lane 5); A1235 astrocytoma cell line (lane 6); M413 normal human fibroblasts (lane 7). *b*, Abelson MuLV *abl*-related transcripts in poly(A)-containing RNAs of cell lines as indicated in *a* were identified by hybridization with 2.5×10^6 c.p.m. ml^{-1} nick-translated 5' *Bgl*II-*Bgl*II DNA fragment of molecularly cloned integrated Abelson MuLV DNA²².

their functional products were analogous to those of the SSV and MC29 transforming genes, the specific transcriptional activation of these *onc*-related genes in certain human tumours may be sufficient to explain the transformed state of the cells.

The present studies used human tumour cell lines rather than primary tumours because of technical difficulties in obtaining sufficient amounts of intact RNAs from the latter. In each case, the tumour cell lines used reproduced the histopathology of the primary tumour in nude mice, indicating that each preserved the transformed phenotype of the original tumour. Our findings of marked differences in the levels of expression of SSV *sis* and MC-29 *myc* transcripts among various cell lines further argue that the increased expression of these genes in only some tumour lines is not attributable to tissue culture adaptation. We have recently examined the expression of retroviral *onc*-related RNAs in a variety of human primary tumours and tumour cell lines of haematopoietic origin⁵² and found the levels of expres-

sion of the different *onc* gene-related transcripts in both fresh and cultured cells to be comparable, with no evidence of alteration associated with tissue culture.

Our demonstration of SSV *sis*-related transcripts in human fibrosarcoma cell lines but not in their normal fibroblast counterparts argues that the expression of this *onc* gene is probably associated with the transformed state of the cells. Moreover, the differences in levels of expression of *sis* or *myc* genes among individual carcinoma or glioblastoma cell lines suggest that the expression of these genes cannot be correlated with a particular tissue type. The more complicated patterns of transcripts related to BALB MSV and Abelson MuLV *onc* genes in human normal and neoplastic cells raise additional questions concerning the mode of processing of such transcripts, and their source from one or more related genes. Nonetheless, the almost universal presence of these transcripts in human cells argues strongly that these viral *onc*-related genes possess some important function(s). Whether they are directly related to the malignant state or to specific stages of cellular differentiation remains to be determined.

Understanding of the mechanisms involved in human neoplastic transformation is still at a very early stage. Recently, Krontiris and Cooper⁴³ and Weinberg and co-workers⁴⁴ provided evidence that cellular transformation can be a consequence of a single dominant genetic change, as transfection with DNA of certain tumours transmitted the transformed phenotype. In other cases, the transformed phenotype has not been transferable^{43,44} or has seemed to be recessive in somatic cell hybrids with normal cells⁴⁵, arguing that the genetic changes associated with transformation may be *trans*-acting or multiple.

One approach to investigating whether the high levels of expression of human cellular analogues of certain viral *onc* genes are aetiologically linked to the development of the neoplastic state extends from the model for transformation associated with dominant genes. We are now analysing DNAs from some of our tumour cell lines for transforming activity in transfection assays. Should these DNAs possess the ability to transform cells, it will be possible to probe such transformants for expression of transcripts of the type specifically elevated in the original tumour cell lines. By such an approach, it may be possible to establish directly whether the high levels of expression of these genes in some human tumours is causally related to the neoplastic state.

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DNA sequences necessary for transcription of the rabbit β -globin gene *in vivo*

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The DNA sequences required for the expression of the rabbit β -globin gene in vivo have been examined. A variety of mutant rabbit β -globin gene templates were linked to a simian virus 40-plasmid recombinant and introduced into HeLa cells; in these conditions the rabbit β -globin gene is expressed from its own promoter. Comparison of the level of β -globin transcripts in a variety of deletion mutants shows that for efficient transcription, both the ATA or Goldberg-Hogness box, and a region between 100 and 58 base pairs in front of the site at which transcription is initiated, are required. Deletion of either of these regions results in a decrease in the level of β -globin transcripts by an order of magnitude; deletion of the ATA box causes an additional loss in the specificity of the site of initiation of RNA synthesis. The DNA sequences downstream from the ATA box, including the natural β -globin mRNA cap site, are dispensable for transcription in vivo.

ALTHOUGH we have a good understanding of the DNA sequence elements which constitute prokaryotic promoters, their eukaryotic counterparts are poorly defined. Comparison of the DNA sequences in the 5' extragenic regions of a large number of genes has revealed two conserved sequence elements with a location consistent with a promoter-like role. The first of these is an A+T-rich region with the canonical sequence 5' TATA_AT_A 3' (the Goldberg-Hogness or ATA box)^{1,2} which is located about 30 base pairs (bp) upstream from the site of initiation of RNA synthesis. The second is the CCAAT box^{3,4} located about 80 nucleotides upstream from the cap site. Considerable evidence now indicates that RNA synthesis initiates at the cap site (see, for example, refs 5, 6).

In vitro transcription systems have recently been developed that synthesize specific capped transcripts from a variety of viral and cellular gene templates^{7,8}—again *in vitro* the site of initiation is the cap site. Using these *in vitro* systems, several groups have shown that the ATA box is required for specific transcription of the adenovirus late^{9,10} conalbumin¹¹ and β -globin¹² genes *in vitro*. Deletion of the CCAAT box has little or no effect on the *in vitro* transcription of these genes⁹⁻¹². The comparative simplicity of the *in vitro* results is not, however, found *in vivo*. In the case of the simian virus 40 (SV40) early region, deletion of the ATA box does not prevent expression of the early mRNAs, and removal of the DNA sequences containing the 72-bp repeat localized at ~150 bp (–116 to –188; and –189 to –261) upstream from the site of initiation of RNA synthesis abolishes early gene transcription¹³. A similar picture is seen for polyoma (R. I. Kamen, personal communication). Likewise, a segment of ~50 nucleotides containing the ATA box is not required for the expression of the sea urchin histone H2A gene when it is injected into *Xenopus* oocytes^{14,15}. However, this deletion does alter the specificity of the site of initiation and causes the appearance of at least three transcripts with discrete 5' ends. This alteration of the specificity of initiation also occurs when the ATA box of the early region of SV40 is deleted¹³. There is also evidence for the role of upstream DNA sequences in the transcription of the histone H2A gene *in vivo*: deletion of a DNA segment containing the sequences from –184 to –524 strongly reduces the level of synthesis from the histone H2A promoter¹⁵.

It is unclear at present to what extent the recent results obtained in the two systems represent the general sequence organization of eukaryotic promoters and to what extent the results are gene specific, or specific to the test system used. There is no obvious sequence homology between the upstream DNA sequences necessary for transcription in SV40 and in the sea

urchin H2A gene that would point to a common promoter element. The SV40 72-bp repeat may represent more than a simple promoter element in the sense that this term is used for prokaryotic promoters (see below).

We have recently examined the *in vitro* transcription of a wide range of deletion mutants of the rabbit β -globin gene in HeLa cell extracts¹². It is important to determine the effects of deletion of these DNA sequences on the expression of this gene *in vivo* and in the same cell type—particularly given the obvious differences recently seen between *in vivo* and *in vitro* transcription in the SV40 and polyoma systems. In addition, we would like to know whether there is any evidence for upstream DNA sequences analogous to those of the SV40 and histone genes in the rabbit β -globin gene system.

To date, two approaches have been used to analyse the transcription of globin genes *in vivo* in animal cells. Mantei *et al.*¹⁶ linked the rabbit β -globin gene to the herpes simplex virus (HSV) thymidine kinase (TK) gene and transformed mouse TK[–] cells to the TK⁺ phenotype. Those cells containing the β -globin gene expressed this at a low, but easily detectable, level. Although this method has the advantage that only a few copies of the β -globin gene are present per cell, the comparison of different transformant cell lines is complex, because the exogenous DNA has become integrated and in each case the site of integration appears to differ. Hamer *et al.* have analysed the expression of the mouse α -globin gene, as an SV40-mouse α -globin recombinant after its introduction into monkey cells permissive for SV40 replication¹⁷. In these conditions the mouse α -globin gene is expressed at a high level from its own promoter. It was recently shown²⁰ that the rabbit β -globin gene inserted (as a 4.8-kilobase (kb) *Kpn*I fragment; see Fig. 1) in the *Kpn*I site of an SV40-pBR322 recombinant is expressed from its own promoter in HeLa cells which are only semi-permissive for SV40 replication. In the present work, we use this SV40-HeLa system to analyse the transcription *in vivo* of a number of deletion mutants of the rabbit β -globin gene.

To assay for the expression of the rabbit β -globin gene, we used S₁ mapping procedures¹⁸, in most cases using as probe a 221-bp *Bst*NI fragment ³²P-labelled at its 5' end using polynucleotide kinase. When hybridized to this probe, rabbit globin mRNA protects a 139-nucleotide fragment from S₁ nuclease digestion (Fig. 1). This variant of the S₁ mapping protocol permits the determination of the 5' end of the respective transcripts to within 1–2 nucleotides¹⁹.

We have analysed the expression of the rabbit β -globin gene at two different sites of insertion in the SV40-plasmid recombinant. The first site is the *Kpn*I site of the origin region of SV40 on the plasmid pSV β G*Kpn* (Fig. 1), that is, the original plasmid analysed by Banerji, Rusconi and Schaffner²⁰. In this case, the rabbit β -globin gene is inserted in the promoter region of SV40.

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In agreement with their original observations, we found that the rabbit β -globin gene is transcribed from its own promoter in these conditions since RNA from HeLa cells infected with this SV40-globin recombinant protects a fragment the same length as that given by normal rabbit β -globin mRNA (Fig. 1).

To investigate whether the rabbit β -globin gene must be present embedded within the SV40 moiety of the recombinant, we first constructed an SV40-pBR328 recombinant by ligating the two respective *Bam*HI-*Eco*RI double digest fragments of SV40 and pBR328 (see Fig. 2). The resultant plasmid, which is analogous to that of Schaffner except that pBR322 is replaced by pBR328, contains the entire early region of SV40, but lacks a 751-bp segment of the late region. We inserted the 2,070-bp *Bgl*II partial digest fragment, which contains the entire rabbit β -globin gene, together with 425 nucleotides of 5'-flanking DNA sequences and 355 nucleotides of the 3'-flanking regions, into the *Bam*HI site of the SV40-pBR328 recombinant. The globin gene segment is therefore interposed between the late region of SV40 and the remaining 3' segment of the tetracycline gene of pBR328. Since this β -globin gene lacks the DNA regions to the 5' side of position -425, we shall refer to this recombinant as ∇ 5' to -425. The rabbit β -globin gene is expressed specifically in both of the two possible orientations (Fig. 1). Clearly then, the globin gene segment does not need to be embedded in the SV40 promoter region. We have estimated the approximate level of β -globin mRNA by standardizing our S_1 mapping procedures with purified rabbit $\alpha + \beta$ globin mRNA (see, for example, Fig. 4). From these data we calculate that $\sim 10,000$ molecules of β -globin mRNA are produced assuming that all cells are expressing the β -globin genes. As the real fraction of cells expressing the gene is likely to be significantly lower than this (usually 15–30% of the cells are positive for SV40 antigen), this value is probably an underestimate by a factor of 2–5.

From the above experiments it can be seen that the rabbit β -globin gene is expressed on an SV40 plasmid in at least two different sites with respect to both the SV40 and plasmid sequences; expression is independent of the orientation of the β -globin gene with respect to the SV40 plasmid DNA sequences. We have obtained similar results with the human β -globin gene (C.K.S., N. Moschonas and E. de B., unpublished) derived from both normal and β^0 -thalassaemic individuals. Although in these cases the early region of SV40 is also present, a functional early region is not required for this expression²⁰.

Rabbit DNA sequences necessary for expression of the rabbit β -globin gene *in vivo*

To determine which rabbit DNA segments are required for the expression of the rabbit β -globin gene in HeLa cells, we have prepared a series of deleted rabbit β -globin genes and introduced these into the SV40-plasmid recombinant. This insertion was performed as described in Fig. 2 legend, and results in the replacement of the pBR328 moiety of the SV40 recombinant with that of pAT153, another pBR322-derived bacterial plasmid. In the first case examined, the rabbit β -globin gene segment extends from -100 to approximately +1,870; the recombinant therefore contains 100 nucleotides of 5' extragenic DNA sequences and ~ 280 nucleotides of 3' extragenic DNA sequences. The plasmid segment (pAT153) is in the opposite orientation to that described above for ∇ 5' to -425 and the rabbit DNA segment is interposed between the 3' segments of the β -lactamase gene of pAT153 and the late region of SV40 DNA (at nucleotide 2,468)^{21,22}. Following the same terminology, we shall call this recombinant ∇ 5' to -100. We have examined the transcripts found in HeLa cells for only one orientation of the β -globin gene (where the 5' end of the β -globin gene adjoins the plasmid sequences), but here again, a high level of rabbit β -globin mRNA is produced with its 5' end

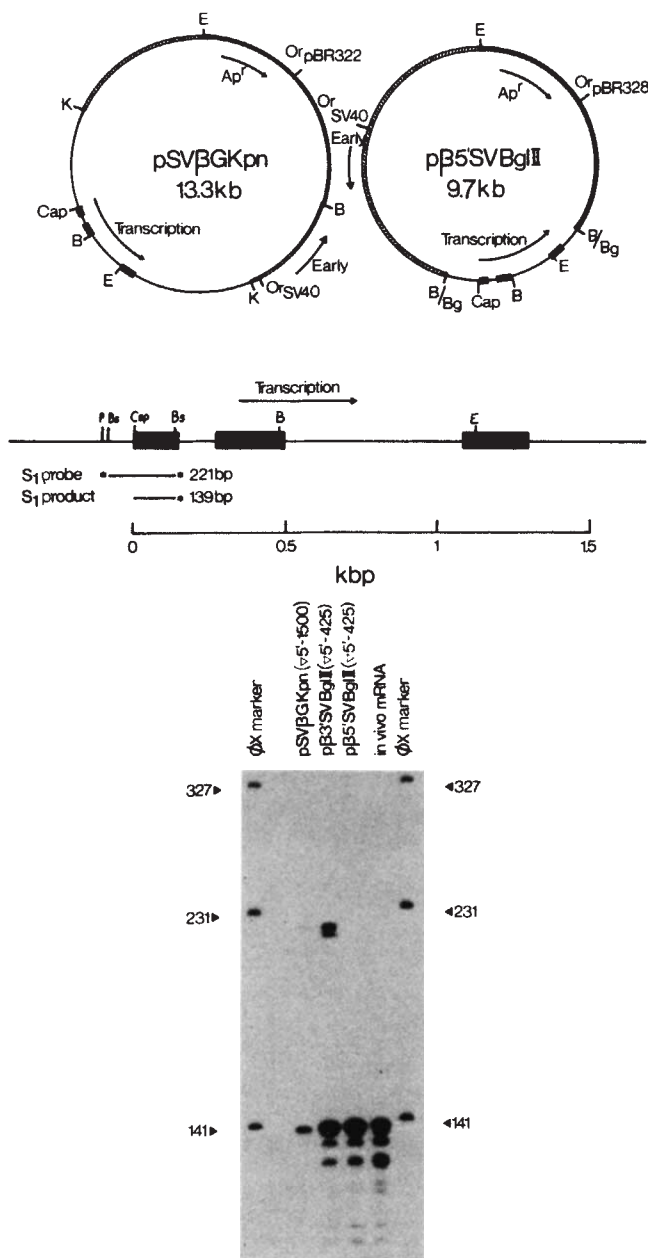


Fig. 1 Transient expression of the rabbit β -globin gene linked to SV40 sequences in HeLa cells. 15 μ g DNA from either pSVBGKpn, pB5'SVBglII or pB3'SVBglII (which has the same structure but the β -globin gene segment is in the opposite orientation with respect to the SV40 sequences) were mixed with 25 μ g of salmon sperm DNA and used to transform a half-confluent 100-mm dish of HeLa cells using the calcium phosphate method²⁷. Three dishes were used for each DNA sample. After 10 h the medium (H21, 10% newborn calf serum) was changed, the cells grown for another 24 h, collected and the RNA isolated by the LiCl-urea method²⁸. The upper part of the figure shows schematic restriction maps of pSVBGKpn and pB3'SVBglII DNA. pSVBGKpn contains the 3.9-kb *Bam*-*Eco* fragment of pBR322, the 4.4-kb *Bam*-*Eco* fragment of SV40 and the 4.8-kb *Kpn*I rabbit β -globin fragment ligated into the *Kpn*I site of SV40 DNA. The construction of pB3'SVBgl and pB5'SVBgl DNA is outlined in Fig. 2—they contain the 2-kb rabbit β -globin *Bgl*II partial fragment in two orientations with respect to the SV40 sequences ligated into the *Bam*HI site of the SV40-pBR328 DNA junction. The rabbit β -globin RNAs present in the transformed HeLa cell RNA were detected by S_1 mapping¹⁸ using end-labelled DNA probes¹⁹. In the centre of the figure is the 221-bp *Bst*NI 5'-labelled fragment used as probe in this experiment, containing 139 nucleotides of the 5' end of the rabbit β -globin gene, which is protected against S_1 nuclease digestion in the DNA-RNA hybrid. The lower part of the figure shows the S_1 products of globin RNAs from HeLa cells transformed with vector DNA, pSVBGKpn DNA, pB3'SVBglII or pB5'SVBglII DNA analysed on a 5% polyacrylamide sequence gel²⁹ (SVpBR328, see Fig. 2). As a positive control the S_1 product of the *Bst*NI probe with *in vivo* rabbit β -globin mRNA is shown. The labelled marker is Φ X174 $\times$ *Hae*III. The numbers on the side are the lengths of the marker fragments in nucleotides. Hatched areas represent SV40 DNA, solid lines plasmid sequences, and thin lines and filled boxes the rabbit β -globin exons.

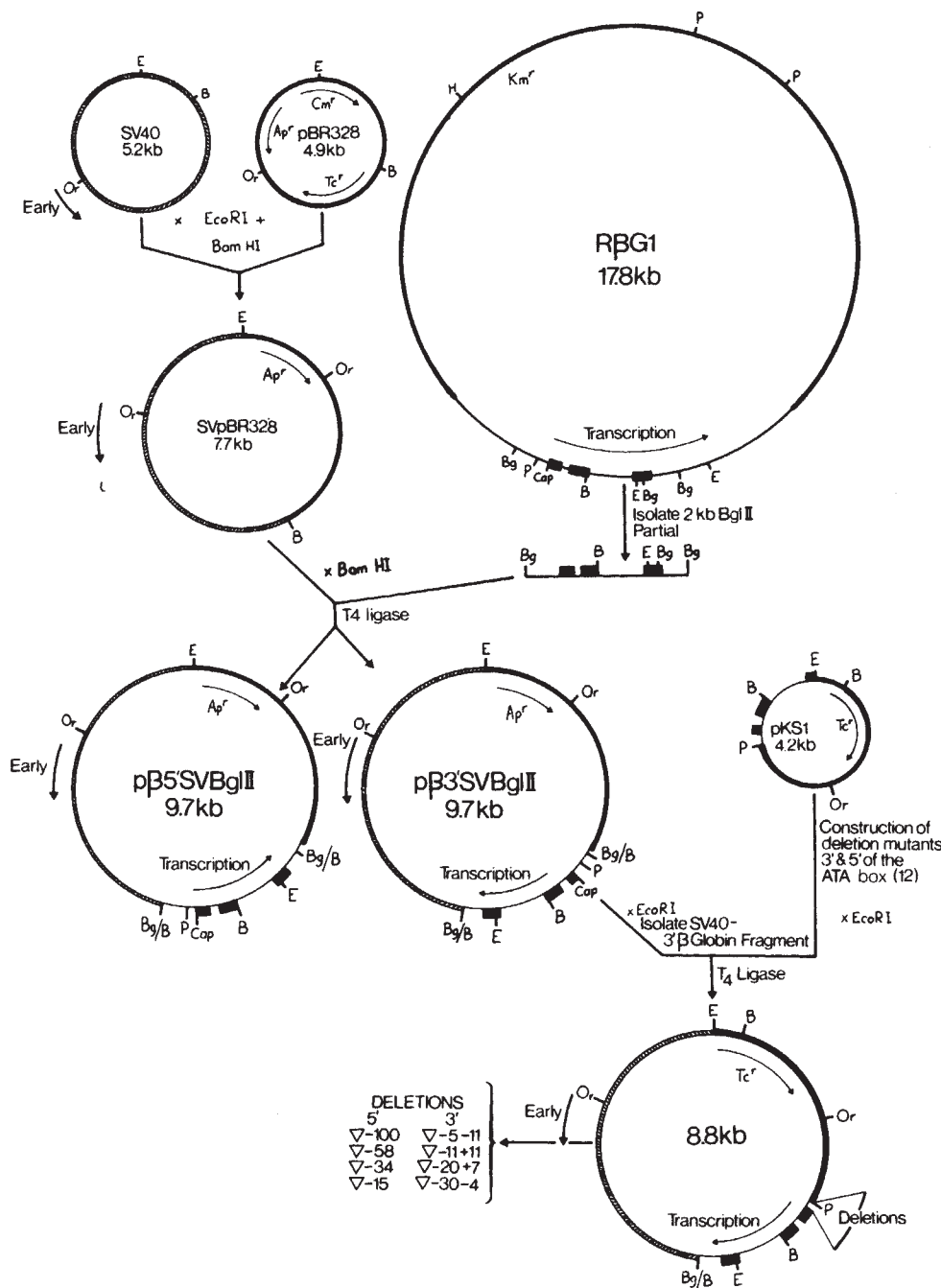


Fig. 2 Construction of SV40 derivatives of β -globin DNAs deleted in the promoter region. To prepare SVpBR328, SV40 DNA and pBR328 DNA (each 5 μ g) were digested with *EcoRI* and *Bam*HI, both large *Bam*-*Eco* fragments were cut out from a 0.5% low melting agarose gel, purified on a DEAE 52 column¹⁹ and ligated for 4 h at 15 °C at a DNA concentration of 20 μ g ml⁻¹ with T4 ligase and transfected into *Escherichia coli* HB101. From the ampicillin-resistant colonies an SV40-pBR328 recombinant was isolated. To clone the 2-kb *Bgl*II fragment (which contains the rabbit β -globin gene) into this vector, 50 μ g RbG-1 DNA were partially digested with *Bgl*II and loaded on a 0.5% low melting agarose gel. The 2,070-bp β -globin *Bgl*II partial band was cut out and the DNA recovered by DEAE cellulose chromatography. This fragment was ligated into the *Bam*HI-linearized pSV328 DNA at a final concentration of 20 μ g DNA per ml. From the ampicillin-resistant colonies both orientations of the globin fragment were isolated with the globin 5' end facing SV40 (p β 5'SVBglII) and the globin 3' end facing SV40 (p β 3'SVBglII) respectively. pKSI DNA, a plasmid containing the *Pst*I-*Eco*RI rabbit β -globin gene fragment (containing the globin sequences from -100 to +1,120) inserted into *Pst*I-*Eco*RI of PAT153¹², was used to produce deletions in the globin promoter region¹² with end points to the 5' side of the ATA box (-100, -58, -34, -15) and the 3' side of the ATA box (-11-5, -11+11, -20+7, -30+4). To clone these DNAs into an SV40-containing vector, 25 μ g 5'p β 5'SVBglII DNA was digested with *Eco*RI and the 4.8-kb SV40-globin band containing *Bam*-*Eco* SV40 and the 3' end of the rabbit β -globin gene was recovered from a 0.5% low melting agarose gel. Then *Eco*RI-linearized pKSI DNA or DNA from the derivative deletion clones was ligated (20 μ g ml⁻¹) into this fragment. Tetracycline-resistant colonies that hybridized to SV40 DNA were picked and screened by restriction analysis. Hatched area, SV40 DNA; solid lines, plasmid sequences; thin lines, rabbit DNA; filled boxes, the β -globin exons.

coincident with that of natural rabbit β -globin mRNA (Fig. 3.). In addition to this component, however, a number of bands are seen which identify the presence of RNAs with 5' ends at several sites from +42 to +48. As these bands are seen when a 5'-labelled 221-nucleotide *Bst*NI minus strand probe is used, these components must be transcribed from the same DNA strand as β -globin mRNA. Together these bands amount to about twice the number of RNA molecules formed from the normal rabbit β -globin promoters. These components are also seen in the case of the ∇ 5' to -425 mutant (that is, the *Bgl*II fragment discussed above), but the abnormal products comprise only about one-third of the total β -globin transcripts. The same abnormal RNAs have been described previously^{23,24} as transcripts produced from the rabbit β -globin gene when introduced into mouse L cells and maintained as stable TK⁺ transformants. We have also synthesized cDNA on RNA from HeLa cells transfected with the rabbit β -globin gene using an *Sfa*NI-*Bst*NI fragment (from the 3' end of the first exon) labelled at its 5' end. This experiment reveals cDNAs corresponding to natural β -globin mRNA and a second predominant cDNA which derives

from an upstream promoter, in addition to several minor shorter cDNA bands which presumably result from early termination of cDNA synthesis. As no cDNA is seen that would correspond to a RNA with a 5' end at +42 to +48, we conclude that the 5' ends detected by S₁ mapping do not result from transcriptional initiation events, but probably derive from upstream RNAs which splice to the A-G at position +47 and +48 of the rabbit β -globin gene. In addition, in S₁ mapping experiments we see a low level of transcripts derived from a plasmid or SV40 promoter.

We have introduced a variety of deletion mutants, derived originally from ∇ 5' to -100, into the SV40-plasmid at the same site, and in the same orientation as the ∇ 5' to -100 mutant. Thus, each mutant is inserted with the 5' moiety of the rabbit β -globin gene adjacent to the 3' regions of the β -lactamase gene. The DNA sequence of each mutant DNA has been determined around the regions spanning the deletion and Fig. 5 shows the relevant areas of sequence.

These DNAs have been introduced into HeLa cells in standard conditions and the 5' ends of the transcripts produced

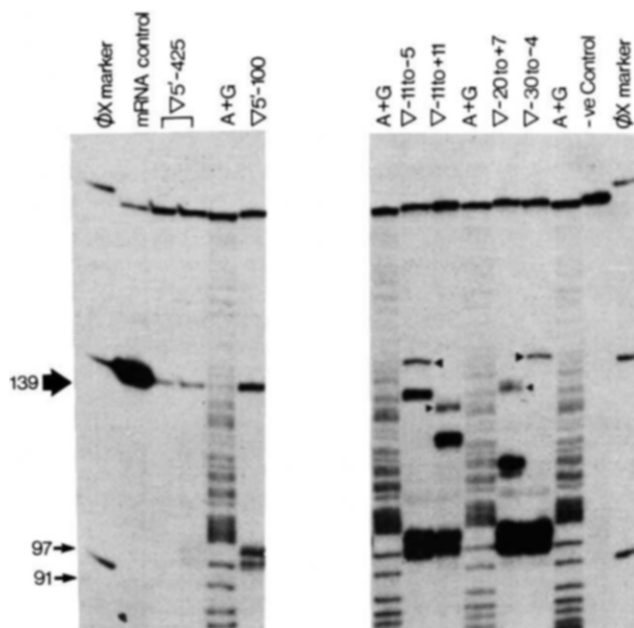


Fig. 3 Transcription of the β -globin gene in mutants at the 3' boundary of the promoter. HeLa cells were transformed with the 5' deletion clones $\nabla -425$ (p β 3'SVgII) and $\nabla -100$ or the 3' deletion DNA clones $\nabla -5-11$, $\nabla -11+11$, $\nabla -20+7$ and $\nabla -30-4$ (see Figs 2, 5) as described in Fig. 1 legend. (See Figs 2 and 5 legends for explanation of the deletions.) The rabbit β -globin mRNAs present in the RNA of those HeLa cells was analysed by S_1 mapping using 5'-end-labelled 221-bp *Bst*NI fragment as indicated in Fig. 1. The S_1 products were loaded onto a 5% polyacrylamide sequence gel and run next to G + A sequence ladders of the 221-nucleotide *Bst*NI minus strand. As an additional size marker, end-labelled Φ X174 $\times$ *Rsa*I fragments were used. The numbers on the side indicate products from the natural β -globin 5' end (139 nucleotides) and RNA products that map within the first exon of the gene (91–97 nucleotides). Besides these two products there are S_1 products labelled with $\blacktriangleright$ in the lanes from the 3' deletion clones which are derived from an upstream promoter as explained in the scheme of Fig. 4.

determined as above. To orient these 5' ends with respect to the DNA sequence of the rabbit β -globin gene, a G + A sequence ladder has been run in every third lane on the same gel (Fig. 3).

Sequence requirements for the regions downstream from the ATA box

We have previously analysed *in vitro* transcription using as templates a variety of mutants which lack DNA sequences to the 3' side of the ATA box. From this we concluded that the DNA sequences downstream from the Goldberg-Hogness box are not necessary for specific *in vitro* transcription because a variety of mutants lacking sequences extending from -20 (or about 4 nucleotides to the 3' side of the ATA box) to as far as $+164$ (in the small intron) are transcribed effectively *in vitro*¹². The *in vivo* cap site is therefore not required; in fact, the RNA polymerase initiates RNA synthesis at about 30 nucleotides downstream from the ATA box, essentially irrespectively of the DNA sequences at the site of initiation. However, extending the deletion to -30 , and hence removing the ATA box, eliminates the specificity of *in vitro* transcripts¹². To determine the effects of the same deletions *in vivo* and in the same cell type, we introduced SV40 recombinants, containing these deletion mutants into HeLa cells. For this purpose we chose the following four mutants: (1) $\nabla -11$ to -5 , which lacks 7 nucleotides between the ATA box and the natural cap site; (2) $\nabla -11$ to $+11$ which has the same 5' break point as the above mutant but which also lacks the natural cap site and a total of 22 nucleotides; (3) $\nabla -20$ to $+7$, which lacks 27 nucleotides and all but 4 nucleotides of the 5' extragenic DNA sequences downstream from the ATA box; and (4) $\nabla -30$ to -4 , which also lacks 27 nucleotides but which, since the deletion is displaced to the 5' side, lacks the ATA box and retains the natural cap site. All these mutants were constructed in the mutant $\nabla 5'$ to -100 and therefore lack the DNA sequences upstream of -100 .

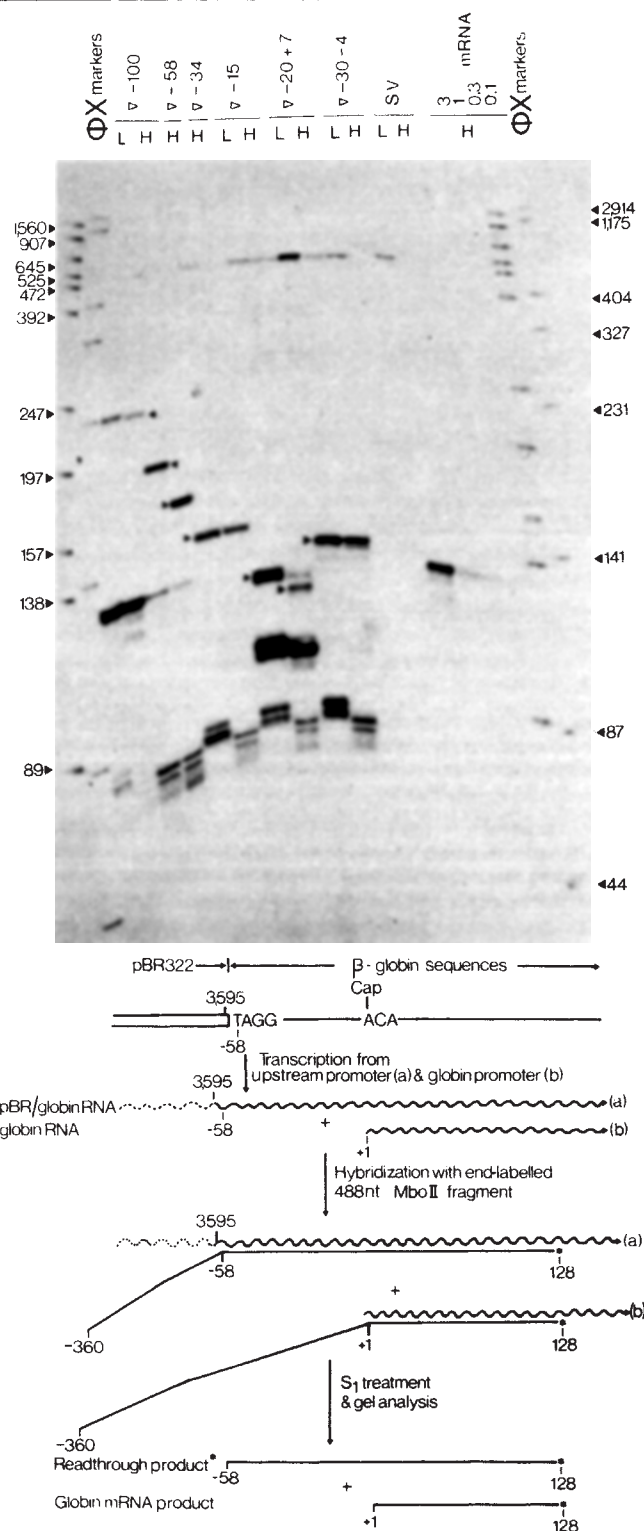


Fig. 4 Transcription of the rabbit β -globin gene from mutant templates lacking DNA sequences at the 5' and 3' boundaries of the promoter. HeLa cells were transformed with 5' deletion clones $\nabla -100$, $\nabla -58$, $\nabla -34$ and $\nabla -15$ and the 3' deletion clones $\nabla -20+7$ and $\nabla -30-4$, as described in Fig. 1 legend. The RNA products from these cells were analysed by S_1 mapping, using a 488-nucleotide 5'-end-labelled *Mbo*II probe that contains 360 bp upstream sequences and the first 128 bp of the 5' end of the rabbit β -globin gene (-360 to $+128$). The S_1 reactions were done at 20°C (L) or 40°C (H) using 3,000 U S_1 nuclease per sample (0.3 ml). The bands labelled $\blacktriangleright$ are derived from RNA starting at an upstream promoter that is picked up by the *Mbo*II probe, as explained in the lower part of the figure. The bands at $+42$ to $+47$ shift at 40°C lower in the gel because S_1 nuclease attacks the A + T-rich splice junction. To calibrate the hybridization, natural rabbit β -globin mRNA (3.1, 0.3 and 0.1 ng) was hybridized and treated with S_1 nuclease at 40°C . Relative amounts of rabbit β -globin mRNAs were determined by scanning bands on X-ray film. Numbers are given in Fig. 5. Labelled marker bands on each side of the gel are Φ X174 $\times$ *Taq*I (left) and Φ X174 $\times$ *Rsa*I (right), respectively. nt, Nucleotides.

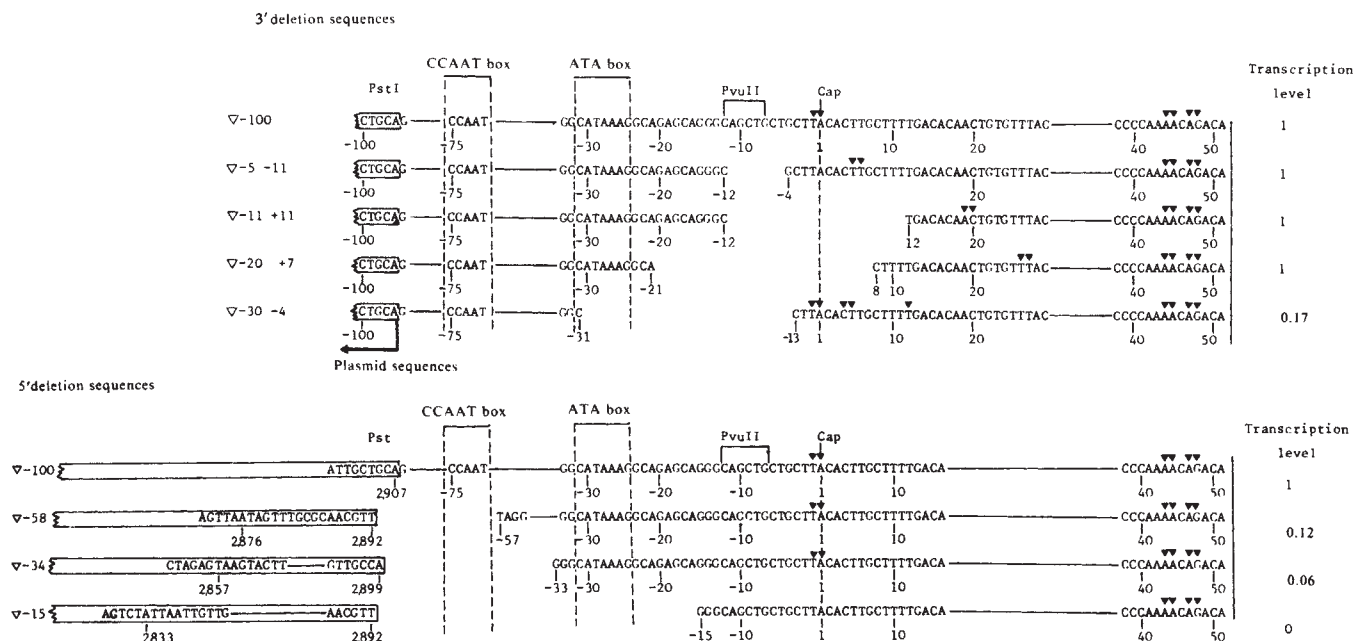


Fig. 5 Sequences of the rabbit β -globin promoter deletions used for SV40-mediated transient expression in HeLa cells. The upper part of the figure gives the relevant areas of DNA (vector-globin junction, CCAAT box, ATA box, cap sequences and start sequences within the gene) of the β -globin clones that have deletions 3' to the ATA box. The size of the deletions are represented by the gap which is left open. As the deletions were constructed from pKSI (Fig. 2), all clones have 5' upstream sequences present to -100 (*Pst*I site). The junctions of vector DNA (pAT153) and rabbit β -globin sequences are indicated by the boxed nucleotides. The lower part of the figure gives the important sequences of the β -globin clones that have been deleted 5' to the ATA box. The size of the deletions is again represented by a gap. All the clones have lost the rabbit sequences 5' to the deletion since the construction of these deletions was started from the *Bal*I site at -75. The vector sequences (pAT153 DNA) present in the clones are indicated by the sequences in the box. The numbers indicated within the plasmid sequences represent the nucleotide which is now at position -75 from the globin cap nucleotide. The 'transcription level' refers to relative levels of globin mRNA in the HeLa cells transformed with these DNAs, which was determined by scanning the autoradiogram of Fig. 4 with a Joyce-Loebl densitometer. The mRNA signals were compared by assuming that the signal of readthrough RNA starting at an upstream promoter is not influenced by the various deletions in the globin promoter region and was therefore taken as an internal standard signal. ∇ Indicates transcription starts seen in the various clones as concluded from the A + G sequence marker lanes in Fig. 3, and the splice sites located at +44 to +48.

The level of specific transcripts from $\nabla -11$ to -5 , $\nabla -11$ to $+11$ and $\nabla -20$ to $+7$ is similar to that seen for the control plasmid $\nabla 5'$ to -100 (Fig. 3). The 5' ends of these mutant transcripts are displaced to the 3' side by essentially the same number of nucleotides as the length of the deletion (Figs 3, 5); we previously found the same result for the transcription of these mutants *in vitro*¹². The same results have recently been obtained for the SV40 early region *in vivo*¹³ and *in vitro*²⁵. These data are consistent with the notion that the polymerase is ultimately bound to the ATA box and starts transcription ~ 30 nucleotides downstream from this site.

The mutant DNA $\nabla -30$ to -4 which has a deletion of the same size as $\nabla -20$ to $+7$, but which lacks the ATA box, shows heterogeneous transcripts, among which is a faint band at the *in vivo* cap site; there are numerous other components. The abnormal RNAs (5' ends at +44 to +48) and 'upstream' plasmid SV40 transcripts are, however, still produced at a similar level to that found for the other mutants. In Fig. 3, a few faint bands are visible in the case of mutant $\nabla -30$ to -4 that are also visible in the control which contains RNA from HeLa cells transformed with the SV40-pBR328 vector; these are products of incomplete S_1 nuclease digestion which are sometimes obtained from this probe when the S_1 nuclease treatment is performed at 20°C; note that these products are seen in the lanes from all RNA samples including the RNA from HeLa cells transformed with only the vector.

To eliminate these incomplete digestion products, we treated hybrids formed between a 5'-labelled DNA probe (in this case a 488-bp *Mbo*II fragment; see Fig. 4) and RNA from HeLa cells transformed with the β -globin gene deletion mutants with S_1 nuclease at 20° or 40°C. Figure 4 shows that the background bands are completely digested at 40°C in these conditions; in addition however, some cleavage occurs near the 5' end of the perfect hybrids formed with β -globin mRNA and the 5'-labelled probe and these overdigestion products are consequently also seen in the hybrids formed with the transcription products of

mutant template $\nabla 5'$ to -100 . More important however, the heterogeneous faint bands seen when the mutant template lacking the ATA box, $\nabla -30$ to -4 , is used, are resistant to S_1 nuclease digestion at high temperature and therefore reflect genuine transcripts. As judged from the S_1 -resistant DNA fragments, transcriptional starts are found at about five sites on this template (see Fig. 5).

Sequence requirements for the DNA regions upstream from the ATA box

The initial experiments with the SV40- β -globin recombinants showed that the rabbit β -globin gene is transcribed effectively when 1,500, 425 and 100 nucleotides of the 5'-flanking DNA regions are present. There are differences in the level of β -globin transcripts found, but as these three examples have been inserted into different sites on the SV40-plasmids and as the plasmid segments differ, the significance of these differences is not clear. (Note that the template with only 100 nucleotides of 5' upstream sequences is transcribed, if anything, better than the two templates with more 5'-flanking rabbit DNA—there is therefore no compelling evidence at this stage to invoke promoter sequences further upstream than -100 .)

To test whether DNA sequences downstream from -100 are required for transcription in this *in vivo* system, we examined the expression of four deletion mutants. A correlation of the transcription efficiency (but see below) with the extent of the 5'-flanking DNA sequences present is justified in these cases as the β -globin gene is present at the same position and in the same orientation with respect to the plasmid and SV40 DNA sequences. The mutant templates we compared are: (1) $\nabla 5'$ to -100 , which contains both the CCAAT and ATA boxes; (2) $\nabla 5'$ to -58 , which lacks the CCAAT box region but retains the ATA box (together with about 30 nucleotides to the 5' side of this structure); (3) $\nabla 5'$ to -34 which also has the ATA box but only 3 nucleotides upstream from this structure; and (4) $\nabla 5'$ to -15 which lacks both boxes.

As stated above, the mutant template ∇ 5' to -100 gives a high level of product from the rabbit β -globin gene promoter, together with a high level of abnormal transcripts which give S_1 nuclease-resistant fragments with ends at +44 to +48.

The level of correct product from the ∇ 5' to -58 mutant is reproducibly reduced when compared with ∇ 5' to -100 (Fig. 4). As these effects are quantitative, rather than qualitative, we have calibrated the levels of correctly initiated RNA chains within the β -globin gene as a function of the deletion of the 5' regions of the β -globin gene in two ways. We have first made use of the fact that there is a plasmid or SV40 promoter which initiates RNA synthesis to the 5' side of the β -globin gene and whose transcripts run into this gene. Since all deletions discussed here are downstream from the promoter, it is reasonable to assume that they will not have a large effect on the frequency of these upstream transcripts. The upstream promoter generates RNAs that form S_1 nuclease-resistant hybrids of a length that in each case reflects the 3' boundary of the deletion in the DNA template, as explained in Fig. 4. We have therefore used this upstream promoter as an internal control; the S_1 nuclease-resistant bands from the upstream promoter are indicated by a $\blacktriangledown$ in Figs 3 and 4. We have compared the levels of products from the two promoters by scanning the autoradiogram; the results are summarized in Fig. 5. Figures 4 and 5 show that the ∇ 5' to -100 template generates significantly more RNA with the correct 5' end, relative to the 'plasmid' promoter, than does ∇ 5' to -58. According to these data, the deletion of the DNA sequences from -100 to -58 caused an eightfold decrease in the level of the correct transcripts. The level of the correct transcripts for the ∇ 5' to -34 mutant is reduced by another factor of 2. Nonetheless, both these mutant templates produce rabbit β -globin RNA with the correct 5' terminus, albeit at reduced efficiency. In contrast, ∇ 5' to -15 shows no detectable product at the correct position. Treatment of hybrids produced with RNA from the HeLa cells transformed with ∇ 5' to -15, with S_1 nuclease at both 20 and 40 °C, gives no detectable S_1 nuclease-resistant fragments except the product from the upstream plasmid/SV40 promoter and the abnormal bands at +44 to +48 (Fig. 4). Thus, while the detection of the DNA sequences upstream from the ATA box reduces the level of correct transcripts in the HeLa cells, a deletion that removes the ATA box alters the specificity of transcription. We have confirmed the quantitative difference in the level of β -globin mRNA from the ∇ -100, ∇ -58 and ∇ -34 mutant templates by transfecting HeLa cells with a mixture of the relevant mutant rabbit and the normal human β -globin genes. The result is the same as that in Fig. 4. The correctly initiated rabbit β -globin mRNA is decreased in amount (relative to human β -globin mRNA) when RNA from the ∇ -100, ∇ -58 and ∇ -34 mutant templates is compared; the 'upstream' RNAs referred to above do not vary in amount relative to the human β -globin mRNA (data not shown).

The quantitative effects of deletion of the sequences to the 5' side of the ATA box may possibly be due to the loss of the CCAAT box region. Inspection of the mutant templates ∇ 5' to -58 and -34 shows that the plasmid DNA sequences, which replace the sequences deleted, do not contain a recognizable CCAAT structure at the usual 40–50 nucleotides upstream from the ATA box. Similar data have recently been obtained by Dierks *et al.*²⁴, who analysed the transcription of the rabbit β -globin gene, linked to the HSV TK gene, after the introduction of mutant templates into L cells and the selection of stable TK⁺ transformants. Their comparison of mutant templates ∇ 5' to -425, ∇ 5' to -76 and ∇ 5' to -68 revealed a high level of the correct β -globin transcripts in the former two cases, but a significantly reduced level in ∇ 5' to -66, that is, on deletion of a region containing the CCAAT box. The ∇ -66 mutant is adjoined to the plasmid DNA sequences by a dG-dC linker tract that is absent in ∇ -76. It is therefore also possible that the difference between the results obtained with these two mutants is influenced by the dG-dC tract²⁴. A requirement for the -80 region for the expression of the human α -globin gene on an

SV40 vector has also been demonstrated (P. Mellon, V. Parker, Y. Gluzman and T. Maniatis, personal communication). It seems that the CCAAT box region may be involved in the efficiency of generation of specific transcripts from the rabbit β -globin promoter. Proof of this awaits the specific alteration of the CCAAT sequence by single base deletion or point mutation experiments.

Conclusions

The results obtained with the rabbit β -globin gene (this work and refs 12, 25) have aspects in common with observations made recently on the sea urchin histone genes after microinjection into *Xenopus* oocytes and the SV40 early genes *in vivo*, but there are also significant differences. First, the ATA box is clearly required for the fixing of the transcriptional start sites of the β -globin gene¹² and the SV40 early genes *in vitro*²⁵ and for all three genes *in vivo* (this work and refs 13, 15). This site of initiation is apparently discrete and is located ~30 nucleotides downstream from the first nucleotide (CATAAAG) of the box. This precision is not influenced *in vivo* (this work) or *in vitro*¹² by the deletion of essentially all the 5'-extragenic β -globin gene sequences upstream from the ATA box (as in ∇ 5' to -34) or all the sequences from the ATA box to past the natural cap site. Thus the positioning of the start site is independent of the CCAAT box region *in vivo* and of the presence (as in ∇ -11 to -5) or absence (as in ∇ -11 to +11) of the natural cap site. These results are broadly similar to those obtained for the SV40 early region¹³.

Deletion of the ATA box generates RNAs with heterogeneous 5' ends for the sea urchin histone H2A gene¹⁴, SV40 early genes¹³ and the rabbit β -globin gene (this work) if the DNA sequences further upstream from the ATA box are present. In the case of the SV40 early region, the amount of early RNA produced does not seem to be strongly influenced¹³, while the ATA box deletion in both the histone¹⁴ and β -globin (this work) genes is a strong down mutation (Figs 3, 5). It is significant that these heterogeneous RNAs are not observed when the β -globin DNA sequences lying between -100 and the ATA box are also deleted, as in mutant ∇ 5' to -15. It is therefore plausible that the CCAAT box region is involved in the promotion of these heterogeneous RNAs when the ATA box is lacking and that their absence in transcripts derived from ∇ 5' to -15 results from the absence of the CCAAT box.

The results for the β -globin gene differ significantly from those for the SV40 early region and sea urchin histone genes, as sequences far upstream from the β -globin gene are not required to generate a high level of β -globin mRNA in the SV40-based system. For both the histone H2A gene¹⁶ and the SV40 early region¹³, sequences located about 200–300 nucleotides upstream are essential for RNA synthesis. This does not appear to be the case for the rabbit β -globin gene since mutant ∇ 5' to -100 is transcribed as well as, or better than, ∇ 5' to -425. There is evidence, however, that DNA sequences near the CCAAT box (which is at -75) are involved in promoting high-efficiency transcription from the rabbit β -globin gene cap site (this work and ref. 25). The SV40 early region contains two putative CCAAT (both CTAAC) boxes at 80 and 101 nucleotides upstream from the first cap site²¹, and deletion of this region causes a decrease in the level of early gene expression. In contrast, the deletion of this region in the sea urchin H2A histone gene stimulates RNA synthesis¹⁴. When considering the effect of DNA sequences far upstream, note that the results with the β -globin gene have been obtained on templates carrying the SV40 72-bp repeats that are absolutely required for SV40 early gene transcription and which constitute the upstream sequences of the SV40 early region. Note also that a region of SV40 which contains the 72-bp repeat is required to obtain a high level of transcription of the rabbit β -globin gene in HeLa cells²⁰. It is therefore possible that this SV40 region is supplanting the requirement for rabbit β -globin upstream sequences; a corollary of this is that these rabbit sequences, if they exist, must not

be present on the 4.8-kb genomic *KpnI* segment which contains the rabbit β -globin gene, since this segment is expressed at a 100-fold level in the absence of the SV40 moiety²⁰. We are currently testing whether such modulator sequences exist further from the β -globin gene. If the SV40 sequences do have this role, then it is significant that this function can be provided by SV40 sequences located at variable distance ($\sim 3,200$ – $4,400$ bp) and in either orientation (at least in 5' to $-1,500$ and ∇ 5' to -425) to the β -globin gene. This is not the type of effect expected from a classical promoter-type sequence. Remember, however, that evidence exists for effects on globin gene expression of deletions far from the genes affected *in vivo* in man (ref. 26 for example). The relevance of these effects to promoter sequences is at present unclear, as is the relevance of the effects

observed in the current type of experiment to the problems of promotion of RNA synthesis from the rabbit β -globin gene in its natural chromosomal location in an erythroblast.

Finally, note that in all the systems discussed here (sea urchin histone genes, SV40 and rabbit β -globin genes), several promoters are being utilized on the same DNA template. This might complicate interpretation: for example, transcription from an upstream promoter might influence RNA synthesis from the promoter being studied, in our case the rabbit β -globin gene promoter. At present insufficient data are available to evaluate the extent of these problems.

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LETTERS TO NATURE

Nature of the shells of NGC1344

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Various theories have been proposed to account for the origin^{1,2} of the low surface brightness features resembling thin shells seen in projection around some otherwise normal elliptical galaxies³. It has proved impossible to discriminate between these models owing to the lack of quantitative data on the nature of the shells. Here we present broad-band optical (*B*, *V*, *R*) and near-IR (*J*, *H*) photometry of one of the shells to determine their constitution and place constraints on their mode of formation. The colour indices derived suggest that the shell comprises a population of stars, perhaps bluer than the main body of the galaxy. There is no evidence for recent star formation. Our results favour dynamical models for the formation of the shells, perhaps from recent mergers of disk galaxies with the elliptical.

The shells are visible on high-contrast derivatives of deep IIIaJ plates taken with the UK Schmidt and Anglo-Australian telescopes (AAT). For quantitative photometry we obtained images with a linear panoramic detector. The instrument available was the UCL Image Photon Counting System (IPCS) at the AAT. The large-scale spatial nonuniformities are worse than a conventional photographic plate and, at the time of our observations, were time variable, perhaps because of the effect of ambient temperature variations on the electronics, so that their removal by flat fielding is not perfect. Also, because of the finite frame rate of the IPCS, data arrival rates above about 30 photons arc s⁻² s⁻¹ will cause saturation. This limits the dynamic range of the system, and standard stars must be very faint. These factors limit the precision of the optical photometry; nevertheless, quantitative data can be obtained.

IPCS images of an area 110×90 arc s containing part of the boundary of the outermost shell to the north-west of NGC1344

were obtained with the AAT on the night of November 11 1980. Figure 1 illustrates the position of the IPCS field. Images of the faint globular cluster Hodge 11 were also obtained, this cluster contains faint electronographic sequences by Walker⁴ and unpublished photometry by Bessell and Gascoigne. Table 1 lists the observations made.

The data were flat-fielded with a combination of exposures of the inside of the dome and the twilight sky. Residual nonuniformities in sensitivity, at the level of 1% in the line increment direction of the IPCS (corresponding to the declination direction on the sky) were removed by fitting a low-order polynomial to the sky background to improve the cosmetic appearance of the images.

To establish zero points the intensity of several star images in the Hodge 11 field was integrated, in a circle of some 5 arc s diameter, and the background level determined from an annulus around each was subtracted. The resultant intensity was compared with Walker's⁴ electronographic magnitudes for *B* and *V*, and in *R* with magnitudes predicted from *V* and *B* – *V* using the colours for giants tabulated by Bessell⁵. Hodge 11 was assumed to be unreddened. This procedure yielded zero points internally accurate to ~ 0.08 in *B* and *V*, and 0.15 in *R*. Extinction coefficients typical for Siding Spring Mountain on a photometric night were adopted. Each of these assumptions will

Table 1 IPCS observations

Object	Filter	Exposure time (s)	Mean wavelength (μm)
NGC1344 shell	<i>B</i>	6,000	0.45
	<i>V</i>	6,000	0.55
	<i>R</i>	8,000	0.66
Hodge 11	<i>B</i>	300	0.45
	<i>V</i>	300	0.55
	<i>R</i>	500	0.66

Conditions were photometric for the whole night.

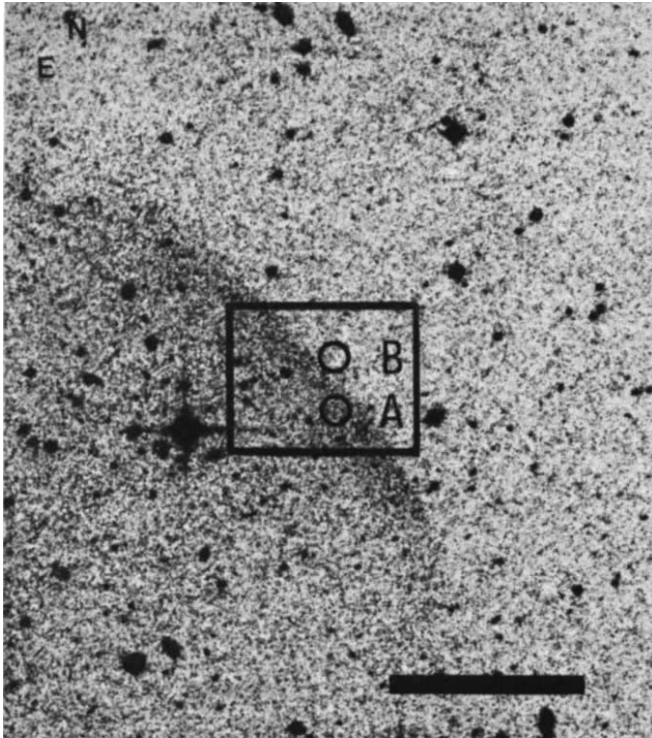


Fig. 1 The north-west shell of NGC1344. IR photometry of the areas indicated by circular apertures A (shell) and B (sky) was obtained. Two-dimensional photometry in *B*, *V*, and *R* was obtained of the area within the rectangle. Scale bar, 2 arc min.

contribute uncertainties of 0.03 mag or less to the derived zero points.

Although the *B* optical image was much more uniform than *V* and *R*, the shell was clearly visible on all three. The shell surface brightness was measured by integrating the intensity in circular apertures of diameters 5–14 arc s on the brighter part of the shell, near the edge, and subtracting from it the intensity in a similar aperture just off the shell, separated from the first aperture in RA only (that is, the direction perpendicular to that along which a polynomial was fitted). This was done at several points along the shell edge, and particularly at the point coincident with the IR aperture (see below and Fig. 1).

Because of the brightness distribution of the shell, conventional IR photometry, using symmetrically placed sky reference beams, is extremely difficult and would, in any case, require a much larger beam throw than was available. We therefore made our observations using the d.c. electronics⁶ of the IR photometer spectrometer on the AAT. The telescope was driven back and forth between the shell and a point 36 arc s north on the sky, completing a cycle shell–sky–shell in typically 5.6 s. At this frequency we could approach quite closely the expected photon shot noise at *J*. The stronger and highly variable sky emission at *H* did not allow such precision. A slight improvement in the precision was obtained by editing from the data any portion where successive measurements (whether of shell or sky) differed by more than 10 times the expected photon shot noise.

Our basic measurements were made with a 14-arc s diameter circular aperture centred at position A in Fig. 1. This and the sky position, B, were chosen to avoid all stars and galaxies visible on a contrast-enhanced Schmidt plate or on the IPCS images. To check that contamination from invisible sources was not influencing our data we made additional observations at *J* with a 10-arc s aperture, and in a second position with the 14-arc s aperture. The consistency of the measured surface brightnesses gives us confidence that our measurement refers to the shell alone.

At *J* the shell was 8×10^{-4} of the minimum sky brightness, which occurred near 3 a.m. LT. At *H* the corresponding value was 1.5×10^{-4} .

Measuring the surface brightness in *B*, *V* and *R* at several points along the shell edge yields:

$$B - V = 1.1 \pm 0.25 \text{ mag}$$

$$B - R = 1.2 \pm 0.3 \text{ mag}$$

Errors due to sky subtraction uncertainties dominate over zero point errors. Surface brightness measured at position A in Fig. 1 are:

$$B (0.45 \mu\text{m}) = 26.5 \pm 0.2 \text{ mag/arc s}^{-2}$$

$$V (0.55 \mu\text{m}) = 25.1 \pm 0.2 \text{ mag/arc s}^{-2}$$

$$J (1.25 \mu\text{m}) = 23.93 \pm 0.10 \text{ mag arc s}^{-2}$$

$$H (1.65 \mu\text{m}) = 23.43 \pm 0.17 \text{ mag/arc s}^{-2}$$

$$B - J = 2.57 \pm 0.22 \text{ mag}$$

$$B - H = 3.07 \pm 0.27 \text{ mag}$$

Because of the long baseline and the lower errors we would rely more on *B*–*J* and *B*–*H* than anything else. All of the measured colours are consistent with those of stars of spectral type between G5 and K4. However, if we are sampling a composite system, we will record bluer stars in the optical than in the IR. Persson *et al.*⁷ report IR and optical photometry for several composite systems. Most are redder than the colours we observe for the shell of NGC1344. For the nucleus of NGC1344 they obtain:

$$B - J = 3.13 \text{ (corrected to match our } J \text{ filter)}$$

$$B - H = 3.84$$

For a small (7 arc s diameter) patch in the galaxy ~50 arc s north of the nucleus we measure:

$$B - V = 0.85 \pm 0.15 \text{ mag}$$

$$B - R = 1.60 \pm 0.15 \text{ mag}$$

Thus the shell near NGC1344 is bluer than the main body of the galaxy.

What do the shells consist of? Observations at 21 cm by A. E. Wright, D.F.M. and D.C. (unpublished) give an upper limit of $7.7 \times 10^8 (50/H_0)^2 M_\odot$ for the neutral hydrogen content of NGC1344 in a 14-arc min beam, which includes the outer shell. Less stringent upper limits were also placed on the H I content of some other shell galaxies. Long slit spectra covering the inner shells of NGC1344 and NGC3923, obtained by P. Quinn and D.C. show no detectable line emission at H β or [O III], even after many thousands of seconds integration time. We conclude that the shells consist almost entirely of cool stars. The colours are consistent with a population of stars which is bluer than the main body of a normal elliptical. Of the systems studied by Frogel *et al.*⁸, some of the bluer lenticulars, such as NGC404 (ref. 8) and NGC5102 (ref. 7), have similar colours. Quinn² shows that features like these can form from mergers of dynamically cool systems, such as disk galaxies, with an elliptical galaxy, so it is perhaps not surprising that the shells have the colours of disk galaxies.

The measured *B* surface brightness implies a total luminosity of the outer shell of $3 \times 10^8 (50/H_0)^2 L_\odot$.

A more remote possibility is that the shells consist of dust, reflecting light from the main body of the galaxy. However, unless the dust grains were abnormally large, their scattering properties would render the shells bluer than the galaxy by at least 1.5 mag over a baseline from *B* to *H*. This is ruled out by the data. Also it has been shown³ that a substantial optical depth would be required to produce the observed shell surface brightnesses, and unless the dust to gas ratio is abnormal, this is not consistent with the limit on the H I content of the galaxy.

In conclusion, the shells apparently consist of stars, probably with the colours of a disk population rather than an elliptical population, but not so blue that they might have been formed very recently, for example in a hot galactic wind. Thus our data support dynamical models for the formation of the shells²,

rather than models in which stars are formed in a galactic wind³, which would predict colours for the shells some 0.5 mag bluer in $B - V$, and therefore some 1.6 mag bluer in $B - J$, than the main body of the galaxy.

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A possible link between the rotation of Saturn and its ring structure

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We present here evidence that two previously unidentified, yet conspicuous gaps in Saturn's rings lie at distances corresponding to 2/3 and 4/3 of the planet's rotation period. Gaps such as these can be produced in a ring of large bodies or small uncharged particles only by a non-axisymmetric gravitational field (both of the above can be associated with the $l = m = 3$ harmonic), a fact that is relevant to models of planetary interiors.

The 'classical' picture of the radial structure of Saturn's rings explains its narrow gaps and boundaries as the consequence of resonant perturbations with certain satellites, in particular, Mimas, Titan and Iapetus. Thus, for example, the inner part of the prominent Cassini division lies at a distance from Saturn (117,900 km) where the orbital frequency of residual ring particles is essentially twice that of Mimas¹. Two recent developments have brought a new perspective to the association between certain ring divisions and satellite resonances. The first is a study by Goldreich and Tremaine² of a mechanism whereby satellites can produce gaps that may eventually achieve widths of hundreds, possibly thousands, of kilometres, while the second is the data provided by the Voyager mission concerning the locations, dimensions and sheer number of ring features³. These new data will require a careful study when more precise positions are provided by Voyager 2. Aspects of this analysis are underway with the preliminary result that many ring features in portions of the ring having low optical thickness ($\tau \leq 0.2$) show a clear correspondence with satellite resonances. There are, however, obvious exceptions in which large gaps fail to have any association with moderate-to-strong satellite resonances. We supply here a possible explanation for two divisions, and specifically for the conspicuous gap, some 200 km wide, located in the outer part of ring C, $86,700 \pm 700$ km from the centre of Saturn. Ring particles at this distance have mean motions one and one-half times the value corresponding to Saturn's 10 h 39.4 min rotation period⁴ and the planet can act to produce a gap, several hundred kilometres wide, just as a satellite would, only if its mass distribution is non-axisymmetric. (The ellipticity of the Earth's Equator is one such example.) If we suppose asymmetries to be present in Saturn, then the disturbing potential⁵ can be written

$$V_{lm} = \frac{\mu_s a_s^l}{a^{l+1}} \sum_{p=0}^l F_{lmp}(i) \sum_{q=-\infty}^{\infty} G_{lpq}(e) S_{lmpq}(\tilde{\omega}, \lambda, \Omega, \theta_s) \quad (1)$$

where l and m are usual subscripts arising in an expansion in spherical harmonics, p and q are introduced by the inclination and eccentricity functions, $F(i)$ and $G(e)$ which allow V to be expressed in terms of orbital elements. The a refers both to the equatorial radius of Saturn, s , and the semimajor axis of a ring particle and $\mu_s \equiv GM_s$. The quantity S_{lmpq} contains the harmonic coefficients, C_{lm} and S_{lm} that are associated with $\cos$ and $\sin$

Table 1 Correspondence of ring features and certain resonances

Resonance	a_{obs} (10^5km)	a_{calc}	Width (km)	n/n_s	
Mimas, 2 : 1	1.179	1.1754	450		
Titan ‘apsidal’	0.773	0.7765	200		
m	q				
2	−1	0.732	0.7201	—	2 : 1
3	+1	1.361	1.3607	~30	3 : 4
3	−1	0.867	0.8637	250	3 : 2

The first, a 2:1 ratio between local mean motion and that of Mimas and the second, equality of local apsidal precession frequency and the mean motion of Titan, provide characteristic values of the positional accuracy of Voyager 1 measurements. Final three (first of these is a boundary, not a gap) are associated with the non-axisymmetric gravitational field of Saturn. n/n_s is the ratio of the orbital frequency at resonance to the planet's rotation rate⁴.

terms whose angular argument is

$$[\lambda(l-2p+q) - m\theta_s - q\tilde{\omega} - (l-m-2p)\Omega] \quad (2)$$

where λ is the mean longitude, $\tilde{\omega}$ and Ω are the longitudes of pericentre and node, respectively, and θ_s is the longitude on Saturn associated with a given harmonic so that $\dot{\theta}_s \equiv \omega_s$ is the spin rate of $810.7601^\circ \text{ day}^{-1}$ (ref. 4). At present we consider only orbits in the equatorial plane so that $i = 0^\circ$, whence it follows that for $F(i = 0^\circ) \neq 0$, $l - m = 2p$. The function G is proportional to $e^{|q|}$, where e is the eccentricity of a ring particle. The case $q = 0$ corresponds (see equation 2) only to synchronous rotation, that is $\lambda \equiv n = \omega_s$, where n is the mean motion. This case—the analogue of the 1:1 resonance of the Trojan asteroids—produces effects that must be analysed differently from those arising at other resonances⁵. Synchronous rotation, presumably not by coincidence, lies in the outer part of ring B where $\tau \approx 1$. The orbital behaviour of (non-colliding) particles near the synchronous distance, d , is well known⁶ so that the surface brightness can apparently show an azimuthal dependence over a narrow annular region centred on d .

The cases, $q = \pm 1$ are the equivalent of the $n/(n+1)$ orbit-orbit resonances. As is evident from the Lagrange planetary equations, forcing terms associated with them contain $e^{|q|-1}$ and are therefore stronger than higher order commensurabilities. We consider only the $|q| = 1$ case so that, for $i = 0^\circ$, the time derivative of equation (2) yields

$$n = \frac{m\omega_s \pm \tilde{\omega}}{m \pm 1} \quad (3)$$

where, apart from $l - m = 2p$, $m \leq l$ is the only remaining restriction on equation (3).

In the saturnian case, the first—and presumably the strongest—nonaxisymmetric term (given by the C_{22} or S_{22} coefficients) produces, apart from the case at the synchronous distance just mentioned, no resonance lying within the ring system. However, the $q = -1$ case, whence $n = 1591.70^\circ \text{ day}^{-1}$ and $a = 72,010$ km lies just inside the inner boundary of ring C, measured by Voyager 1 at $73,200 \pm 700$ km. (All these calculations use $a_s = 60,330$ km, $J(\propto C_{20}) = 0.0244488$ and $K(\propto C_{40}) = 0.0034375$ and $\mu_s = 3.79209 \times 10^{22}$.)⁶ Two cases where the identification seems likely arise when $m = 3$, that is $n = (3\omega_s - \tilde{\omega})/2$ and $n = (3\omega_s + \tilde{\omega})/4$. Calculations (Table 1) place the former at 86,370 km, the latter at 136,070 km and, in each case, especially the former, a prominent ring gap is nearby. A caveat remains because systematic errors as large as ± 700 km (ref. 7) attend the positional measures made by Voyager 1 and the accurate Voyager 2 results require reductions not yet complete. To offset this uncertainty, we have compiled in Table 1 predicted and measured locations for two strong satellite resonances whose identifications are well-established, together with those, just considered, that we associate with the planet's rotation.

It seems premature to examine evidence linking less conspicuous gaps in ring A with larger values of m . For future efforts

and because much 'fine structure' has been detected in the Cassini division⁷, we list in Table 2 selected resonance locations arising from non-axisymmetric terms up to $m = 13$. The stellar occultation experiment of Voyager 2 will provide the observational material for comparison.

Using the analysis of Goldreich and Tremaine², we can estimate the magnitude of the C_{33} coefficient required to produce the large gap in outer C ring by replacing the disturbing function due to an exterior satellite by one appropriate for the non-axisymmetric mass distribution of Saturn. For the case $i = 0^\circ$ and $|q| = 1$, the disturbing function (1) can be written.

$$V_{|q|=1}^{lm} = (-)^{(l-m)/2} \frac{\mu_s}{\pi^{1/2} a_s} 2^{m-1} \left(\frac{a_s}{a}\right)^{l+1} \Gamma\left(\frac{l+m+1}{2}\right) \times (l-2qm+1)eS_{lmq} \quad (4)$$

where Γ is the gamma function and S_{lmq} contains the C_{lm} and S_{lm} coefficients. Equation (4), when combined with the development of Goldreich and Tremaine, shows that a combined harmonic coefficient, $(C_{33}^2 + S_{33}^2)^{1/2} \approx 2 \times 10^{-10}$ is sufficient to generate the observed gap width if the process² can operate essentially for the age of the Solar System. As there is no evidence favouring such long-term operation, it is useful to see if the lower limit can be raised. Values of $(C_{33}^2 + S_{33}^2)^{1/2}$ must be large enough to ensure that a gap can be cleared, thanks to angular momentum transport, in the presence of continuing viscous diffusion. The relevant equations have been derived by Goldreich and Tremaine⁸, whence the most stringent condition is

$$(C_{33}^2 + S_{33}^2)^{1/2} \approx \frac{1}{3} \left(\frac{\nu}{\Omega_K r^2} \right)^{1/2} \quad (5)$$

where ν is the kinematic viscosity and Ω_K the local keplerian angular velocity. The former has recently been derived⁷ from observations, made by Voyager 1, of the Cassini division, $\nu = 170 \pm 80 \text{ cm}^2 \text{ s}^{-1}$, which approximately accords with theoretical estimates². If we assume that the same value of ν applies in parts of the ring having larger optical thickness, then the right-hand side of equation (5) yields $\sim 3 \times 10^{-8}$ as the appropriate limit.

The gap at $1.361 \times 10^5 \text{ km}$ in the outer part of ring A is produced by the same $l = m = 3$ harmonic, with $q = +1$ rather than -1 (although higher harmonics may contribute to both). Equation (4) indicates why it is so much narrower (see Table I). In addition to the difference in the saturnicentric distance of the two, for the outer resonance, the factor $(l-2qm+1)$ drops by a factor of 5.

Following the Saturn encounter of Voyager 2, a more detailed examination of the positions and widths of all ring features, especially in regions of low optical thickness, is necessary. We shall also need an equally careful review of the mechanism of gap formation to infer more precise values for the harmonic coefficients. There now seems to be some evidence that Saturn's gravitational field does contain at least the (3,3) harmonic. Although it is tempting to suppose that the (2,2) term, with its

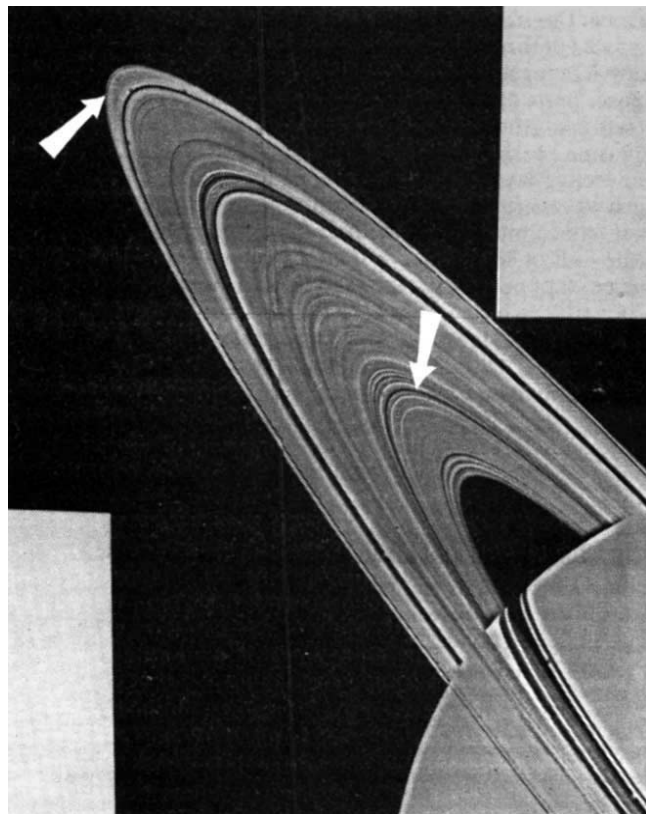


Fig. 1 Ring system from Voyager 1. Arrows mark the two gaps that can be accounted for if Saturn has an $l = m = 3$ harmonic.

strong resonance just inside the inner ring C boundary is responsible for the truncation of that ring, how this happens is not clear. If the ring system once did extend closer to Saturn, into the D-ring region, then were the planet to have a substantial (2,2) term, a broad gap near 72,000 km could be produced, with its outer boundary at the observed truncation of the C ring. However, we would apparently still require an additional process⁹ to reduce the surface density inside that resonance. In this context, we should note that the effective inner boundary of Jupiter's ring lies at a distance corresponding to the $l = m = 3$ resonance, or, where $2n = 3\omega_1 - \dot{\omega}$. Adoption of $\omega_1 = 870.5443^\circ \text{ day}^{-1}$ (corresponding to a period of 9 h 55 min 29.37 s), $J = 0.02208$, $K = 0.00244$ and $R_1 = 71430 \text{ km}$ shows this resonance to be located at $1.227 \times 10^5 \text{ km}$, whereas, although the ring can be faintly traced nearly to the jovian cloud tops, measurements place the inner edge of the bright portion (viewed in backscattered light) at $1.228 \pm 0.007 \times 10^5 \text{ km}$ (ref. 10). The influence on particle orbits that we attributed to tesseral harmonics of the gravitational field might instead result from the primary's inclined, displaced magnetic field. As for Saturn, the outer ring boundary seems to be established by a small satellite.

It seems unlikely that harmonic coefficients with $m \neq 0$ refer to the shape of Saturn's (probable) rock-ice core. However, Stevenson¹¹ has proposed a new interior model with three distinct layers, in addition to the core, that can account both for the planet's unexpectedly weak magnetic field and its excess heat flux. The model's outermost layer consists of an envelope of molecular H_2 and He, with the latter somewhat depleted. The existence of metallic H characterizes the next layer which is inhomogeneous because of the limited solubility (dependent on temperature and pressure) of He in metallic H. Deeper, droplets of He eventually dissolve to form a much more homogeneous layer above the core. The inward migration of He releases gravitational energy and might inhibit convection, but, for our purposes, the vital point is the existence of several distinct

Table 2 Positions (in optically thin regions) of additional strong resonances associated with higher harmonics of Saturn's field

m	q	a_{calc}	n/n_s
4	+1	1.3037	4:5
5	+1	1.2690	5:6
8	+1	1.2159	8:9
9	+1	1.2060	9:10
10	+1	1.1980	10:11
11	+1	1.1915	11:12
12	+1	1.1860	12:13
13	+1	1.1814	13:14

Cassini division, where many narrow gaps appear⁷, stretches from ~ 1.177 to $\sim 1.210 \times 10^5 \text{ km}$. Precise positions from Voyager 2 are necessary for a detailed comparison.

layers. The likelihood that various instabilities can exist on the surfaces of these layers such that their interfaces assume a wavy form was suggested by R. Smoluchowski. In a nonlinear regime, denser parts of the fluid will collect near the troughs, while the crests can grow by buoyancy to reinforce the wave. Although diffusion seeks to eliminate such waves, they may, in a poorly convective layer, persist for a long time or be regenerated. If such waves are the source of Saturn's gravitational asymmetries, then concomitant ring features may well vary to some time scale—all of which means that any estimate of $(C_{33}^2 + S_{33}^2)^{1/2}$, but perhaps not its existence should be treated cautiously. As an alternative to instabilities at layer interfaces, one might consider an interior model possessing large scale convective cells that develop and sustain non-axisymmetric mass distributions—presumably over extended periods of time.

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Radiation effects and the leach rates of vitrified radioactive waste

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There is much concern that long-lived radioactive elements incorporated in glasses for the disposal of highly active nuclear waste might eventually return to the environment. The most important mechanism begins with leaching of the glass by groundwater and a leach rate, usually based on laboratory tests of simulated vitrified radioactive waste, has been used as the basis of analyses of the radiological safety of a waste repository^{1–3}. The possibility that radiation damage to the glass from the products of nuclear decay could change the leach rate has been studied both by the incorporation of active elements in the glass and by irradiation from external sources. The most important contribution to radiation damage comes from the recoil nuclei during α decay⁴. Typically the recoil nucleus has a kinetic energy of ~ 100 keV and displaces $>1,000$ atoms in the glass. Experiments which simulate this damage by incorporating short-lived α -emitting isotopes such as ^{238}Pu into the glass have not shown significant increases in leach rate at doses equivalent to $>10^{18}$ α decays g^{-1} (refs 5–9). Figure 1 shows that for wastes considered in the UK this corresponds to a time after vitrification of at least 10^3 – 10^4 yr. Recent work with ion beams inducing the radiation damage^{10–12} has suggested that large increases (up to a factor of just over 50) in leach rate will occur after a critical dose of radiation from the α -decay processes. In addition, experiments in which the leaching solution is irradiated with γ rays^{13–15} have shown that radiolysis effects in the water can increase the leach rates of glasses. We present here new data and calculations which relate these studies to the conditions expected in a real waste repository. Our analysis is described in more detail elsewhere^{16,17}.

Dran *et al.*^{10,11} irradiated glasses and crystalline minerals with 200-keV Pb ions and found that an enhanced leach rate occurs

Table 1 Leach tests on samples of glass 189* doped with $^{238}\text{PuO}_2$

Date	Dose (α -disintegrations g^{-1})	Equivalent time (yr)	Holding temperature ($^{\circ}\text{C}$)	
			First year	Subsequent years
			50	170
			20	20
			Leach rates ($\text{mg cm}^{-2} \text{ day}^{-1}$)	
November 1975	0.89×10^{18}	7,000	1.6	1.5
February 1977	2.0×10^{18}	250,000	2.3	2.3
March 1977	2.1×10^{18}	300,000	2.4	2.2
November 1977	2.7×10^{18}	500,000	2.3	2.6
July 1978	3.3×10^{18}	700,000	2.3	2.5
January 1981	5.5×10^{18}		3.2	2.7
February 1981	5.6×10^{18}	1,400,000	3.0	

The equivalent times assume 0.5% of the plutonium and 0.25% of the uranium originally present in Magnox fuel go into the waste. The fuel is assumed to be reprocessed after 6 months out of the reactor. Leach rates were measured by the Soxhlet method, in which the sample is in contact with frequently changed distilled water at 100°C . The initial Soxhlet leach rate expected for undoped glass of this composition is $1.3 \pm 0.2 \text{ mg cm}^{-2} \text{ day}^{-1}$.

* The composition of this glass in wt% is $\text{SiO}_2:41.5$; $\text{B}_2\text{O}_3:21.9$; $\text{Na}_2\text{O}:7.7$; $\text{Li}_2\text{O}:3.7$; simulated waste product oxides: 25.2.

above a critical fluence of $\sim 5 \times 10^{12}$ ions cm^{-2} , which corresponds to the dose at which the individual damage zones (each of lateral extent ~ 10 nm) produced by the Pb ions overlap. Such effects have been observed before, for example, in ion-irradiated garnets¹⁸. One expects similar overlap of damage zones to occur in a glass containing α emitters at a dose of $\sim 10^{18}$ decays g^{-1} .

Each incident 200-keV Pb ion in the ion beam experiments produces $\approx 3,000$ displacements. The irradiated region has a depth of 50 nm so that at a critical fluence of 5×10^{12} ions cm^{-2} the average number of displacements is not more than $3 \times 10^{21} \text{ cm}^{-3}$. We calculate that each α decay produces 1,380 displacements so the critical fluence is equivalent to 2.2×10^{18} α decays cm^{-3} . For a typical glass density of 2.6 g cm^{-3} this is 8.5×10^{17} decays g^{-1} . Dran *et al.*¹¹ quote $\sim 2 \times 10^{18}$ decays g^{-1} as the equivalent dose to a Pb ion fluence of 5×10^{12} ions cm^{-2} . Their method is based on an equivalent number of heavy ion tracks crossing unit surface area. However, whichever approach is used the appropriate figure is clearly $\sim 10^{18}$ decays g^{-1} .

Table 1 shows recent results from work at Harwell on samples of UK glass 189 which have been doped with 5 wt% of $^{238}\text{PuO}_2$. Regular leach tests of these samples in distilled water using the Soxhlet test have been made since their manufacture in 1975. Only modest increases in leach rate have been observed even after 5.6×10^{18} α decays g^{-1} . The PuO_2 may not be completely homogeneously dispersed in these samples, but it is believed that most of it is in solid solution in the glass as would be the case for the α -emitting isotopes in real vitrified waste. There is no evidence of a large increase in leach rate as found by Dran *et al.* One of these samples has now also been tested in a 250 g l^{-1} NaCl solution at 100°C as used by Dran *et al.* and showed an increase by a factor of only 1.2 over an unirradiated sample. Furthermore the leach rates were an order of magnitude smaller in the NaCl solution than in distilled water.

We therefore believe that the large effects noted by Dran *et al.* do not occur in the simulations using α emitters, which, of course, are accelerated versions of the effects that will actually occur in practice. In trying to rationalize these results we have considered possible effects of dose rate¹⁶. Simulations with α emitters are carried out at rates 10^4 – 10^5 times higher than those pertinent to a real waste repository at times ≥ 100 yr, whereas the ion beam experiments are accelerated by a factor of 10^{10} – 10^{11} . If $r \text{ cm}^{-3} \text{ s}^{-1}$ is the rate of damaging events and $v \text{ cm}^3$ the volume of a damaged zone created by one event (that is, by a single incident ion or single recoil nucleus), then the time required to reach a stage where the zones overlap is $t_{\text{crit}} \approx (rv)^{-1}$.

Obviously if an individual zone recovers (anneals) in a time short compared with t_{crit} , overlap of zones is not reached. By extrapolating data obtained at Harwell on the annealing of density change of the glass and the release of irradiation-induced stored energy on heating, we estimate¹⁶ that the mean recovery time of a damaged zone is in the range 10^4 – 10^7 s between 25 and 200 °C. With $v \sim (10 \text{ nm}^3)$ we estimate that $t_{\text{crit}} \sim 10$ s for the ion beam experiments, $\sim 10^8$ s for simulations with α emitters and $\sim 10^{10}$ – 10^{12} s for a real waste glass. We therefore suggest that the effects observed with ion beams will not occur in simulated or real glasses in which the damage results from α -decays at a relatively low rate.

Our remarks apply more strongly to the work by Hirsch¹² in which the damage is produced by 2-keV Ar ions. These have a range in the glass of only 2.4 nm and accelerate the damage rate by a factor of perhaps 10^{14} . Such low energy ion beams also produce changes in surface composition and (at high fluences) topography^{19–21} which may have more influence on chemical attack than the effects of structural changes produced by radiation damage in the bulk of the material.

Some recovery of damage may also occur during laboratory leach tests if these are carried out at 100 °C as in the Soxhlet method. We estimate that up to 15% recovery could have occurred during leach tests for the samples in Table 1. Obviously recovery effects must be considered both during irradiation and during any tests after irradiation; our estimates show that they could be extremely important in the long time, low dose-rate conditions that apply to real vitrified waste.

McVay and co-workers^{13–15} have measured the leach rates of US glass 76–68 while the glass and aqueous leachant were being irradiated with ^{60}Co γ rays at a dose rate of 2.4 Mrad h^{-1} . They found increases by a factor of about two in the rate of loss of weight of the glass and larger increases in the leach rate of some individual elements. They showed that these effects are not due to radiation damage to the glass by γ rays, but must be associated with the effects of radiation on the leachant. In those experiments where air was present, some of the effect could be caused by the production of nitric acid when air or nitrogen is irradiated in the presence of water^{22,23}. However, the increases in leach rate in a system in which air was removed¹⁵ must be caused by radiolysis of the water.

To relate these experiments to the conditions likely to exist in a waste repository, we have used the methods of radiation chemistry^{24,25} to calculate the concentrations of radicals produced by the irradiation of water next to the glass surface. These calculations consider the known yields^{26–27} of the primary species H^+ , e_{aq}^- , H , OH , HO_2 , H_2 and H_2O_2 produced by ionization of water, and known rates of secondary reactions²⁸ involving these species, water molecules and possible solutes. We have made preliminary calculations for the following irradiation conditions. (a) 2.4 Mrad h^{-1} γ radiation, as used by McVay *et al.* (b) 4 Mrad h^{-1} α radiation, which represents the situation for leach rate measurements on samples doped with $^{238}\text{PuO}_2$ (see Table 1), where simultaneous leaching and irradiation is inevitable. (c) The radiation conditions in a repository, assuming pessimistically that the leachant contacts the glass directly at all times. Some typical dose rates at various times after vitrification are given in Table 2.

Table 2 Typical radiation dose rates to the leachant in a waste repository

Time after vitrification (yr)	Dose rates (Mrad h^{-1})	
	Lightly ionizing radiation (β , γ)	α radiation
4	5.3×10^{-1}	2.72×10^{-2}
50	8×10^{-2}	2.25×10^{-2}
500	~ 0	1.08×10^{-2}

These figures assume that the leachant is in contact with the radioactive glass and are based on the same data as in Fig. 1.

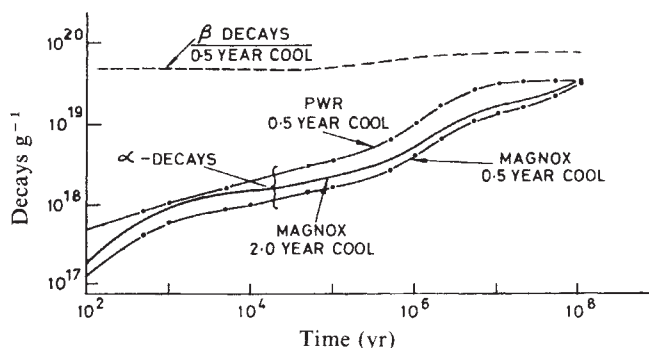


Fig. 1 The number of decays g^{-1} as a function of time for highly active vitrified waste: 12.5% fission products in glass; 0.5% Pu, 0.25% U to highly active vitrified waste. The different curves refer to different reactors and processing conditions as indicated. Each α decay produces over 1,000 atomic displacements whereas each β decay produces ≤ 1 displacement. Long term radiation damage effects are therefore dominated by α decays.

Because dissolved oxygen was removed in McVay and Pederson's experiments¹⁵ and is not expected in an underground repository, most of our calculations are for sealed systems with no gas phase present. However, in case (b) we assumed that the water is saturated with dissolved air at the temperature used as was the case in these experiments.

The results are shown graphically in Fig. 2. We note first that in case (a) the radicals with highest concentration are OH and O_2^- , suggesting that these are the species responsible for the enhanced leach rates in McVay *et al.*'s experiments (note that McVay and Pederson¹⁵ show that H_2O_2 has no discernible effect). We find small changes (factor of ≤ 2) in the concentrations of H^+ and OH^- . Experiments^{29,30} in which leach rate has been measured as a function of pH show that such small changes will have a negligible effect on leach rate. The absence of any significant change in initial leach rate for $^{238}\text{PuO}_2$ -doped glasses when compared with undoped glass (see Table 1) suggests that OH is more active than O_2^- in promoting an increase in leach rate, since we find in Fig. 2 that there is a higher O_2^- concentration in case (b) than in case (a). Clearly in Fig. 2, in real repository conditions the concentrations of these radicals fall to values low compared with those for the laboratory experiments in case (a). We also find that the presence of the solutes NO_3^- and Cl^- , which are found in real waters equilibrated with rocks, can reduce the OH concentration by factors of up to several thousand. We conclude that radiolysis of the leachant will not have a large effect on the leach rate in a real repository.

We have tried to quantify the production of nitric acid when air or nitrogen is irradiated in the presence of water. From experimental data^{22,23} we deduce that the nitric acid concentration $N \text{ mol dm}^{-3}$ at time $t \text{ h}$ for irradiation of a sealed air/water system is given by

$$N = 2C_0R[1 - \exp(-1.45 \times 10^{-5} G D t)] \quad (1)$$

where D is the dose rate in Mrad h^{-1} , R is the ratio of the volume of air to the volume of liquid and the G value for the reaction is 1.9. C_0 is the concentration of N_2 in the air in mol dm^{-3} . Equation (1) shows that a pH change from 5.7 to 3.3, as observed by McVay and Pederson¹⁵ after 5 days irradiation in the presence of air, would be consistent with a value of R of the order unity.

A repository should be free of air pockets soon after burial (N. A. Chapman, personal communication) and the absence of air as a separate phase precludes the formation of nitric acid because this compound is a negligible product of the irradiation of water containing dissolved nitrogen^{23,31}. Measurements by Rai *et al.*³² with particulate suspensions of plutonium compounds suggest that nitric acid could be formed fairly efficiently from dissolved nitrogen, but this interpretation is suspect as air was present in their tubes as a gas, which was probably irradiated by the Pu-containing particles, distributed

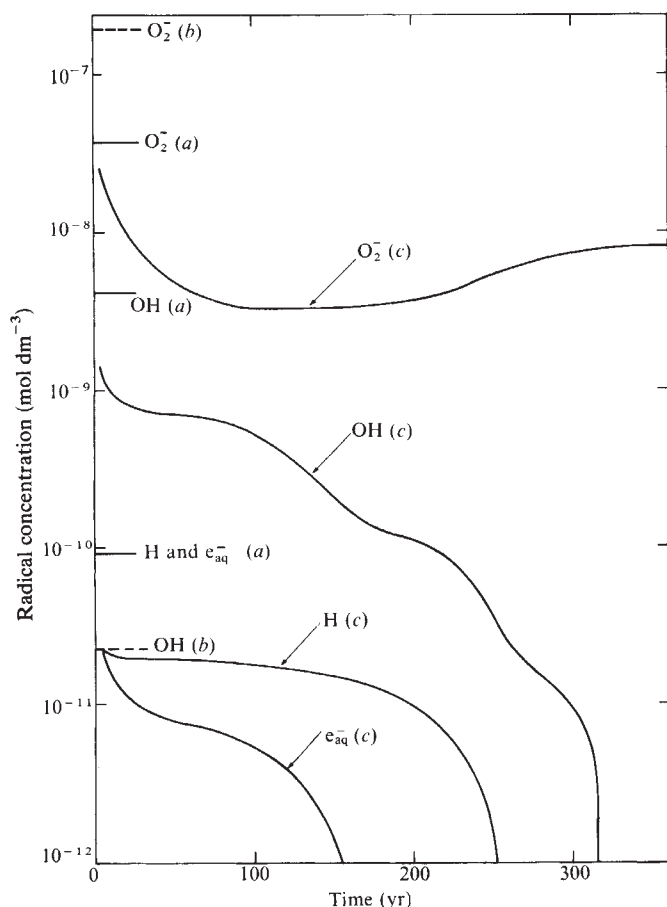


Fig. 2 Concentrations of the radiolytic species H, OH, e_{aq}^- and O_2^- produced by radiolysis of the water next to glass in a repository. The concentrations for irradiation conditions (a) and (b) described in the text are shown on the left. The H and e_{aq}^- concentrations in case (b) are below the scale at 1.1×10^{-13} and 4.1×10^{-14} , respectively. The rise in the O_2^- concentration after ~ 100 yr is caused by the preponderance of α radiation at long times (see Table 2).

non-uniformly (the greatly increased energy yield found with shaking, and the increase in the rate of acid formation with time support the latter interpretation).

If air were present in a repository the radiation could reach it only through containment and probably a layer of water. At times when the containment may have failed, say after a few hundred years, Table 2 shows that the only significant radiation is from α particles. These have a range of $<4 \times 10^{-2}$ mm in water, so would be completely absorbed in this thickness. The conditions in a repository therefore militate quite strongly against the formation of nitric acid and its consequent contribution to leaching.

The experimental data discussed here all point to leach rates in real repository conditions no more than a few times larger than those of unirradiated glasses. Note, however, that rather wide extrapolations and interpolations in dose rate are involved, and more data are needed over a range of dose rates. Leaching data for specific elements would also be informative because nearly all existing measurements refer to total weight loss. Experiments that have been done suggest that actinide elements dissolve more slowly than the bulk glass structure^{14,33}.

Note also that the leach rate as usually measured in laboratory tests is unlikely to be the rate-determining factor for the dissolution of glass in a real waste repository, because of the very slow flow rate of water past the glass surface³⁴⁻³⁶. In these conditions the rate of removal of elements from the glass will be controlled by the saturation solubility of the glass in the water, or, in stagnant conditions, by the diffusion of species away from the glass surface¹⁷. Chapman *et al.*³⁶ have estimated that the

water flow through a (fractured) 1 m^3 block of glass in a repository even without clay backfill would be $\sim 4 \text{ ml day}^{-1}$. If we assume a solubility of 2 g l^{-1} , which is an order of magnitude higher than the solubility of amorphous silica in water at 50°C (ref. 37), then the rate of mass loss of the block will be 8 mg per day . If the block is fractured into 10-cm cube segments the total surface area is $6 \times 10^5 \text{ cm}^2$ so that the effective leach rate would only be $10^{-8} \text{ g cm}^{-2} \text{ day}^{-1}$. This can be compared with laboratory values (see Table 1) and the figure of $10^{-7} \text{ g cm}^{-2} \text{ day}^{-1}$ below which radiological analysis gives a clear margin of safety². Backfilling might reduce the flow rate and hence the effective leach rate by a factor³⁶ of 10^4 . A major uncertainty in this argument is the effective solubility of real vitrified waste, but there is evidence that the solubility of actinide species from glasses is extremely low³⁸. Radiation damage should not change the solubility by more than a small factor as one is dealing with a situation approaching chemical equilibrium, rather than with a kinetically determined rate process as in a normal surface leach rate measurement.

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A mesospheric source of nitrous oxide

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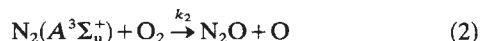
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In the terrestrial atmosphere, nitrous oxide (N_2O) has a major role in the chemistry of ozone. Current atmospheric models assume that N_2O is produced only by fixation at the Earth's surface and that there are no local sources in the stratosphere or mesosphere. We point out here that a significant *in situ* N_2O source does exist above 20 km due to the excitation of the metastable $\text{N}_2(\text{A}^3\Sigma_u^+)$ state by resonance absorption of solar UV photons that penetrate deeply into the atmosphere through the $\lambda 1,800\text{--}2,200 \text{ \AA}$ $\text{O}_2\text{--O}_3$ window. This source significantly affects the NO altitude distribution in the mesosphere and, in the Earth's prebiological atmosphere, made N_2O an important stratospheric constituent.

Literature estimates of the profile of eddy diffusion coefficients, of the effective lifetime of N_2O in the lower atmosphere and of the magnitude of the role of nitrous oxide in the chemistry of the ozone layer are predicated in part on the assumption that there are no significant *in situ* N_2O sources. Previously, the only aeronomically interesting mechanism known to produce nitrous oxide was the reaction¹

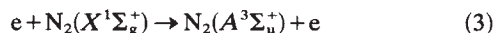


But the rate of this reaction in the gas phase is negligibly slow, so that it is not a factor at stratospheric altitudes and above². However, a recent laboratory experiment found that the reaction³



produces nitrous oxide with an efficiency of $0.6 \pm 30\%$. Because reaction (2) is the dominant $\text{N}_2(\text{A}^3\Sigma_u^+)$ loss mechanism below 90 km, the resulting N_2O production rate in the mesosphere and below will be directly proportional to the local $\text{N}_2(\text{A}^3\Sigma_u^+)$ excitation rate.

Metastable $\text{N}_2(\text{A}^3\Sigma_u^+)$ molecules are formed efficiently⁴ by electron impact



with a total cross-section that is quite large ($\sim 1.4 \times 10^{-16} \text{ cm}^2$ at 15 eV). In the upper atmosphere, therefore, these energy-rich molecules will be produced copiously by the secondary electrons resulting from the absorption of relativistic electrons and solar protons in the mesosphere⁵ and from the absorption of auroral electrons⁶ and solar X rays and EUV in the ionospheric D and E regions.

Processes (2) and (3) may result in N_2O concentrations as high as 10^9 cm^{-3} in the auroral D region during an active substorm⁶.

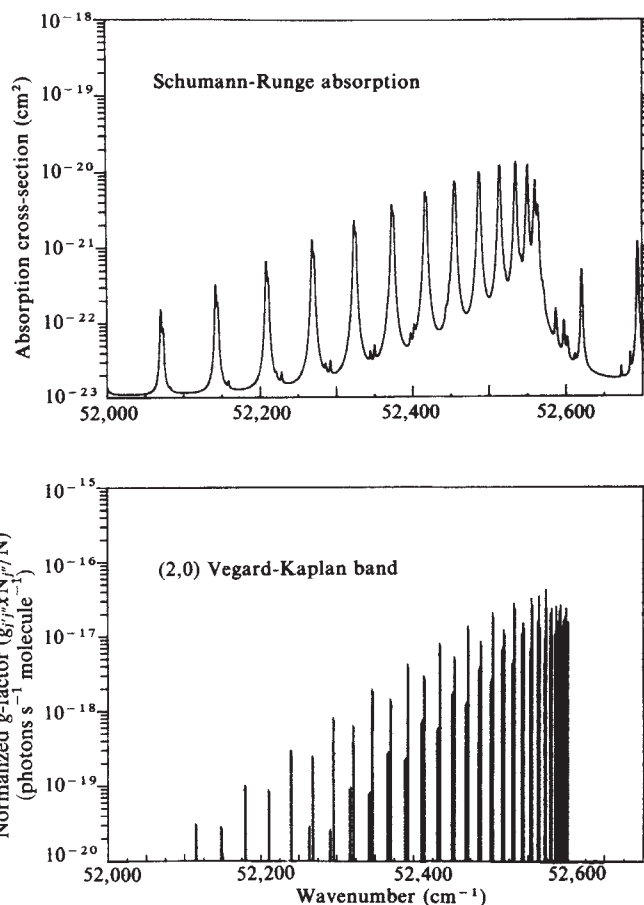
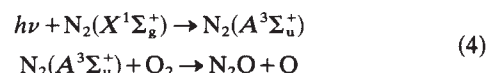


Fig. 1 The normalized g -values $[g_{J'J''} \times N_{J''}/N]$ for the (2,0) Vegard-Kaplan band $[\text{A}^3\Sigma_u^+ \leftrightarrow \text{X}^1\Sigma_g^+]$ and the absorption cross-sections for the (5,0), (6,1), (8,1) and (9,1) Schumann-Runge bands of O_2 .

However, energetic particle precipitation is a less important $\text{N}_2(\text{A}^3\Sigma_u^+)$ source mechanism at mid-latitudes and in the equatorial region and at low altitudes ($z < 50 \text{ km}$). In these regions, optical pumping of the $\text{A}^3\Sigma_u^+$ state by the resonant absorption of solar photons in the $\lambda 1,800\text{--}2,100 \text{ \AA}$ wavelength range



seems to be an effective nitrous oxide source throughout the mesosphere and to altitudes as low as 20 km in the stratosphere.

At first sight, optical pumping of the $\text{N}_2(\text{A}^3\Sigma_u^+)$ state by solar UV photons looks unpromising: the $\text{A}^3\Sigma_u^+$ state is metastable with an average radiative lifetime of 2 s (ref. 7), so that the cross-sections for resonance absorption will be quite small. Furthermore, the Franck-Condon factors for the $\text{X}^1\Sigma_g^+ \rightarrow \text{A}^3\Sigma_u^+$ transition favour transitions from high vibrations levels ($v'' > 4$) of the $\text{X}^1\Sigma_g^+$ ground state which are negligibly populated at the low temperatures that prevail in the stratosphere. However, from a stratospheric point of view, these properties could be regarded as an advantage because the solar

Table 1 The $g_{J'J''}$ factors for solar optical pumping of the metastable $\text{N}_2[\text{A}^3\Sigma_u^+(v', 0)]$ state

$^1\Sigma_g^+ - ^3\Sigma_u^+$	(0, 0)	(1, 0)	(2, 0)	(3, 0)	(4, 0)
$R_2(J'')$	$5.04(-18)R^*$	$2.39(-17)R^*$	$4.22(-16)R^*$	$2.03(-15)R^*$	$5.34(-15)R^*$
$Q_1(J'')$	$2.52(-18)$	$1.20(-17)$	$2.11(-16)$	$1.01(-15)$	$2.67(-15)$
$Q_3(J'')$	$2.52(-18)$	$1.20(-17)$	$2.11(-16)$	$1.01(-15)$	$2.67(-15)$
$P_2(J'')$	$5.04(-18)P^\dagger$	$2.39(-17)P^\dagger$	$4.22(-16)P^\dagger$	$2.03(-15)P^\dagger$	$5.34(-15)P^\dagger$

* For example, $5.04(-18)R = 5.04 \times 10^{-18} [J''/(2J'' + 3)]$ photons per s per molecule.

† For example, $5.04(-18)P = 5.04 \times 10^{-18} [(J'' + 1)/(2J'' - 1)]$ photons per s per molecule.

photons that optically pump the Vegard–Kaplan band system [$X^1\Sigma_g^+ \leftrightarrow A^3\Sigma_u^+$] will penetrate deeply into the atmosphere before they are absorbed. Thus, the net $N_2(A^3\Sigma_u^+)$ production in the 20–50 km region of the lower atmosphere will reflect the outcome of the competition between process (4) and the absorption of solar photons by O_2 and ozone.

The Vegard–Kaplan bands that contribute most significantly to nitrous oxide production in the mesosphere and stratosphere are the (0, 0), (1, 0), (2, 0), (3, 0) and (4, 0) transitions (see Table 1) which are located quite favourably in the 1,800–2,100 Å [O_2 – O_3] atmospheric window.

We have estimated the magnitude of the local $N_2(A^3\Sigma_u^+)$ production rate due to solar UV absorption as follows. The excitation rate of a specific rotational transition (J', J'') for a ($v', 0$) Vegard–Kaplan band, η , can be expressed in terms of the g -factor⁸

$$\eta(z; v', 0; J', J'') = g(v', 0; J', J'') N_{J''}(z) \quad (5)$$

where $N_{J''}(z)$ is the population density of the $N_2(X^1\Sigma_g^+)$ ground state molecules in the $v''=0$ vibrational level and in the J'' rotational level at altitude z , and

$$g(v', 0; J', J'') = \pi F_\nu(z) \frac{\pi e^2}{mc} f(v', 0; J', J'') \quad (6)$$

In this expression, $f(v', 0; J', J'')$ is the optical oscillator strength

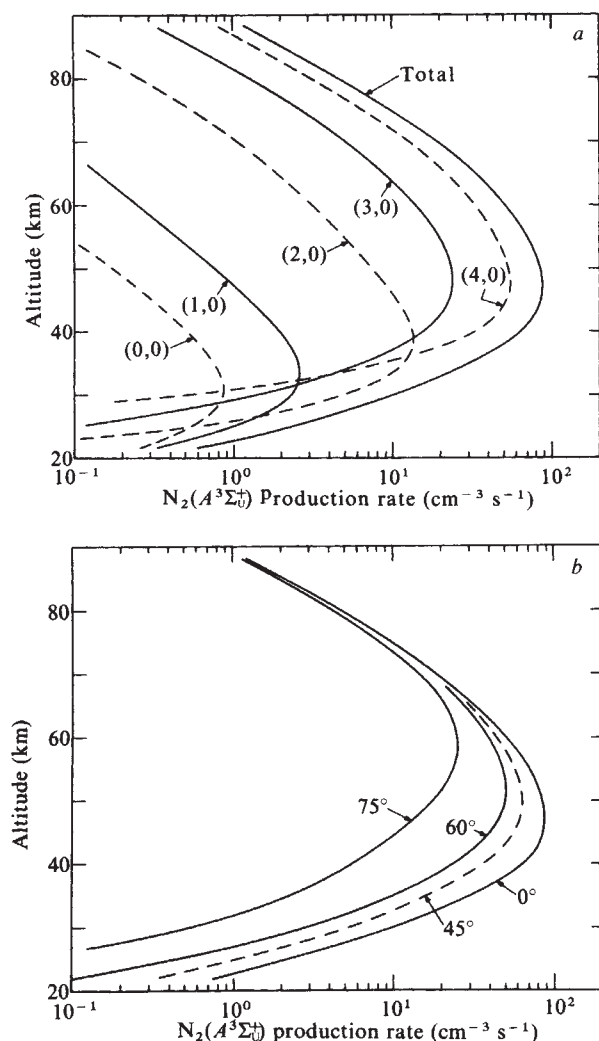


Fig. 2 *a*, Production rates of $N_2(A^3\Sigma_u^+)$ due to solar resonant scattering for overhead Sun conditions. The number in the parentheses denotes the vibrational levels in the $A \leftarrow X$ electron transition. The curve 'total' is obtained by summing the contribution from the various vibrational levels. *b*, Total production rate of $N_2(A^3\Sigma_u^+)$ for various solar zenith angles.

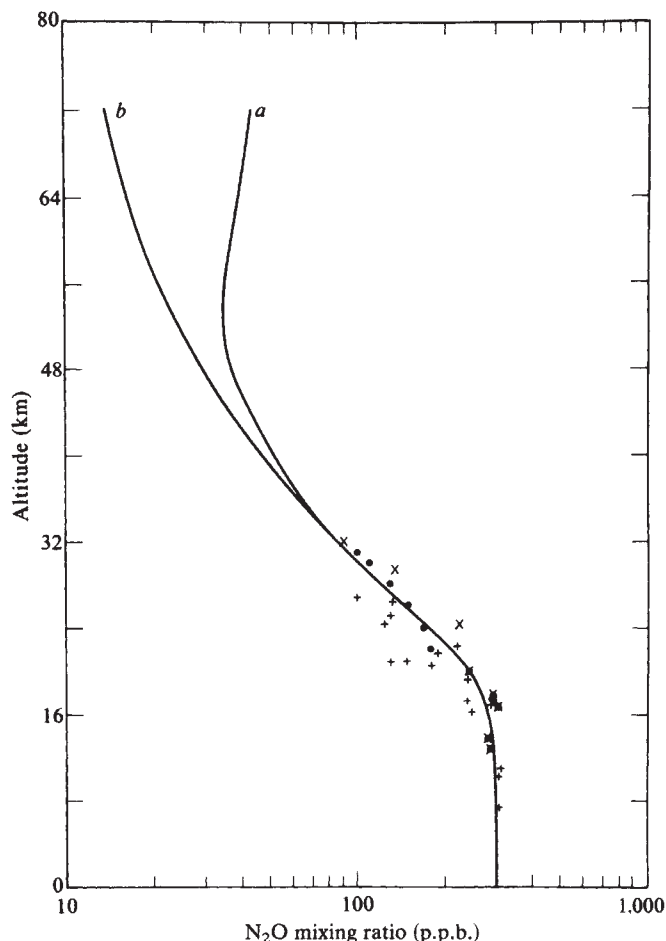


Fig. 3 Mixing ratios of N_2O . Curves *a* and *b* represent theoretical values for two cases: with and without the nitrous oxide production due to the excited $N_2(A^3\Sigma_u^+)$, respectively. Observed values are from Farmer *et al.*¹⁴ (●), Schmeltekopf *et al.*¹⁶ (+) and Ehhalt *et al.*¹⁵ (x).

of the $J'' \rightarrow J'$ rotational transition of the ($v', 0$) VK band, and $\pi F_\nu(z)$ is the incident photon flux at frequency, ν , from the Sun. The local flux can be related to the flux outside the atmosphere, $\pi F_\nu(\infty)$, in units of photons $cm^{-2} s^{-1} Hz^{-1}$ by the expression

$$\pi F_\nu(z) = \pi F_\nu(\infty) \sum_i \exp(-\tau_\nu^i / \mu_0) \quad (7)$$

where τ_ν^i / μ_0 is the slant optical thickness due to absorption. In this calculation, only O_2 and O_3 were considered as significant absorbers. For modelling purposes, the calculations assumed an effective rotational temperature of 230 K and used the solar flux data published by Nicolet⁹.

Values for $f(v', 0; J', J'')$ were derived from the work of Shemansky⁷ for the R_2 , P_2 , Q_1 and Q_3 branches of each of the five VK bands [$(v', 0); v' = 0 \rightarrow 4$] included in this study by using the expression

$$\frac{\pi e^2}{mc} f(v', 0; J', J'') = hc \nu B_{J''}^{0, v'; J'} \quad (8)$$

The effective optical thickness of the atmosphere due to O_3 absorption was calculated using the cross-section data of Hudson¹⁰ and the ozone profiles given by Nicolet⁹. The effects of O_2 absorption in the Schumann–Runge bands and in the Herzberg continuum were treated in detail. This involved calculating the effective absorption cross-section for each rotational line of the Vegard–Kaplan bands due to the rotational lines of the 14 branches: $P_1, P_2, P_3, R_{P31}, P_{P13}, Q_{12}, P_{Q23}, R_{Q32}, R_{Q21}, R_1, R_2, R_3, P_{R13}, T_{R31}$. In this computation, the absorption oscillator strengths and line widths given by Lewis *et al.*¹¹ were used.

Figure 1 shows a representative result for the (5, 0) Schumann–Runge band and the (2, 0) Vegard–Kaplan band. The specific $g(v', 0; J', J'')$ values were then calculated from these results using the solar flux data reviewed by Nicolet⁹.

Figure 2a shows the production rate for the first five vibrational levels of $A^3\Sigma_u^+$ state due to solar optical pumping for a zenith angle of 0° . At 50 km, the effective N_2O production rate is $\sim 51 \text{ cm}^{-3} \text{ s}^{-1}$ assuming a 60% efficiency for reaction (2) (see ref. 3). Based on current one-dimensional photochemical models, the nitrous oxide loss rate due to photodissociation and reactions with $O(^1D)$ atoms would be approximately $79 \text{ cm}^{-3} \text{ s}^{-1}$ and $8 \text{ cm}^{-3} \text{ s}^{-1}$, respectively, at 50 km. Thus, optical pumping of the $N_2(A^3\Sigma_u^+)$ state by solar UV photons is a significant N_2O source in the mesosphere. Figure 2b shows the net $N_2(A^3\Sigma_u^+)$ and N_2O production rates for several solar zenith angles.

To explore the effects of this source, we carried out a globally averaged one-dimensional calculation in which the N_2O photochemistry and transport were treated in detail. The model assumes that N_2O molecules are lost by photodissociation and by reaction with $O(^1D)$ and formed by process (4). The $O(^1D)$ concentration was calculated on the basis of a local chemical equilibrium between its production from O_3 photolysis and loss by quenching collisions with N_2 and O_2 . The empirical method of Shimazaki *et al.*¹² was used to determine the absorption of solar UV in the Schumann–Runge bands. We assumed an N_2O mixing ratio of 3×10^{-7} at the lower boundary and a zero flux at the upper boundary at 100 km. Out of the two profiles of eddy diffusion coefficients recommended by Danielson¹³, we used the k_1 profile because it gives a better performance in describing the available observations of N_2O . Calculations were done for a solar zenith angle of 30° using the US Standard Atmosphere 1976. Two cases were considered: (a) with and (b) without the process (4). Results are shown in Fig. 3, together with the observed values of N_2O mixing ratios from Farmer *et al.*¹⁴, Ehhalt *et al.*¹⁵ and Schmeltekopf *et al.*¹⁶. Because observations of N_2O above 30 km are of low precision they have been omitted. The fact that we reproduce the observed values of N_2O mixing ratios verifies the soundness of our basic model and computational details.

An important point from our analysis is that the new source of N_2O is capable of sustaining significantly increased amounts of N_2O in the upper stratosphere and mesosphere. Compared with the case (b), which ignores the process (4), N_2O mixing ratios are increased by 10%, 39%, 102% and 192%, respectively, at 40, 50, 60 and 70 km in the case (a). Nitrous oxide is a source of nitric oxide (NO) through the reaction:



Accordingly, increased amounts of N_2O in the upper stratosphere (30–50 km) will lead to increased NO production in this region. In the present calculation, the height-integrated production rate of NO in the 30–50 km region increased by 10%. The observed amounts of NO are less than the predictions of contemporary theories¹⁷ which neglect process (4). Thus, the new source of N_2O (process (4)) magnifies the current discrepancy between the observed and predicted amounts of NO in the upper stratosphere. The key point, however, is that the introduction of this source should lead to a more realistic assessment of this discrepancy and to its satisfactory solution.

Mesospheric NO has been attributed to either the downward transport of NO from the ionospheric E region or to the upward transport from the stratosphere, because there is no *in situ* source of nitric oxide in the mesosphere other than the indirect and weak production by reaction (9). Thus, depending on the transport parameters used, theoretical models had difficulties in accounting for the observed amounts of mesospheric NO (ref. 18), such as those from Tisone's¹⁹ experiments. It is, therefore, significant that the introduction of our new source leads to 100–200% increases in the mesospheric production of NO by reaction (9). Possibly, this increase could alleviate the difficulties of accounting for the observed mesospheric NO.

Finally, note that the magnitude of the N_2O source due to

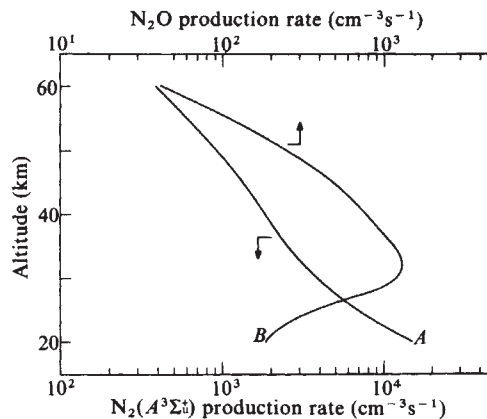


Fig. 4 The $N_2(A^3\Sigma_u^+)$ and N_2O production rate due to solar optical pumping the $A^3\Sigma_u^+$ state 3,500 Myr ago in the Earth's prebiological atmosphere.

process (4) is modest in our present-day atmosphere because O_2 and O_3 absorption below 35 km severely attenuates much of the solar flux that would otherwise optically pump the $N_2(A^3\Sigma_u^+)$ state. However, in the Earth's prebiological era ($\sim 3,500$ Myr ago), the O_2 and O_3 content of the atmosphere was many orders of magnitude smaller so that copious N_2O production occurred. Figure 4 shows the results of a simplified calculation illustrating the potential magnitude of this source using the prebiological model atmosphere 1 of Kastings and Walker²⁰.

During this period, the $O(^1D)$ concentration was also very small so that reaction (9) was not a significant N_2O sink; photodissociation and transport were the chief loss mechanisms. In the present-day stratosphere, only solar photons with wavelengths $>1,750 \text{ \AA}$ figure prominently in N_2O photodissociation and result in an effective J_∞ value $\sim 10^{-6} \text{ s}^{-1}$. However, with the loss of the O_2 – O_3 screening effects in the prebiological atmosphere, more energetic solar photons could reach the upper stratosphere where they would, at first sight, accelerate the N_2O photodissociation. But the absorption by N_2O of most photons with wavelengths $<1,560 \text{ \AA}$ leaves the N_2 photofragment in the $B^3\Sigma_g^-$ or $A^3\Sigma_u^+$ state which subsequently form N_2O through reaction (4), so the effective N_2O photodissociation loss rate at low altitudes is not enhanced much over present-day values. This suggests that the N_2O concentration at 30 km in the Earth's prebiological atmosphere was of the order of 10^9 cm^{-3} during a period well before bacterial action provided a surface source. Ultimately, as the oxygen content of the atmosphere increased, reaction (9) gradually began to provide an atmospheric source of fixed nitrogen (NO) which may have played an important part in the subsequent development of life.

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Auroral effects on power transmission line systems

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As human activity advances northwards, its supporting systems, such as power transmission lines and oil/gas pipelines, extend across the auroral zone. As the auroral electrojet, a concentrated ionospheric current along the auroral zone, induces a potential of up to 1 V km^{-1} on the Earth's surface, a significant amount of electric current can be induced in long conductors which are grounded at points separated by a long distance¹⁻⁶. We show here that auroral activity can induce surges in the protective relay system of a power transmission line.

We have previously reported that major fluctuations in a power transmission line (138 kV, 100 A) near Fairbanks are mostly induced by auroral activity. The Healy-Fairbanks power transmission line, which runs approximately in the north-south direction ($\sim 166 \text{ km}$), is terminated at an autotransformer at the Gold Hill substation which is located just outside the city of Fairbanks. Aurora-induced current fluctuations in the power line were monitored in terms of the autotransformer 'neutral current' by measuring the voltage developed across a $62.5 \mu\Omega$ resistor inserted between the autotransformer neutral and substation ground.

Figure 1 shows both the 'Earth current' and the neutral current records which were obtained on 3 October 1981. The former was obtained by recording the voltage developed between two electrodes (separated by 100 m) which are located about 5 km from the autotransformer site. Most of the fluctuations in the Earth current were caused by the simultaneous auroral activity¹⁻⁷. Comparing both records, the earth-current fluctuations are well reproduced in the neutral-current fluctuations, indicating that the latter were mostly induced by auroral activity.

To examine possible damaging effects of the aurora-induced currents on the powerline system, however, we need to determine whether such low-frequency (or quasi-d.c.) fluctuations affect the protective relay system and the autotransformer in the circuit. The autotransformer has an electrically isolated winding which provides a current to drive protective relays. This current is zero in a perfectly balanced three-phase power system and is non-zero if an imbalance (such as a short-circuit or other unbalanced load) occurs. An additional cause of increased relay current is an enhancement of certain current harmonics (third, ninth, fifteenth and so on). Half-cycle saturation of transformers caused by d.c. winding current produces such harmonics.

Figure 1 shows the simultaneous record of the current in the protective relay system. The impulsive changes superimposed on continuous and random fluctuations of the neutral current are indicated by arrows. Those impulses were associated with significant low-frequency (or quasi-d.c.) excursions of the neutral current. Figure 2 shows another example of the simultaneous recordings of the current in the protective relay circuit and the neutral current during a medium intensity geomagnetic storm of 19 December 1980. A series of surges can easily be identified in the relay current. We believe that these results are the first confirmation that auroral activity induces surges in the protective relay system of a power transmission line.

D.c. transformer excitation causes a larger than normal amount of harmonic components to be generated in a trans-

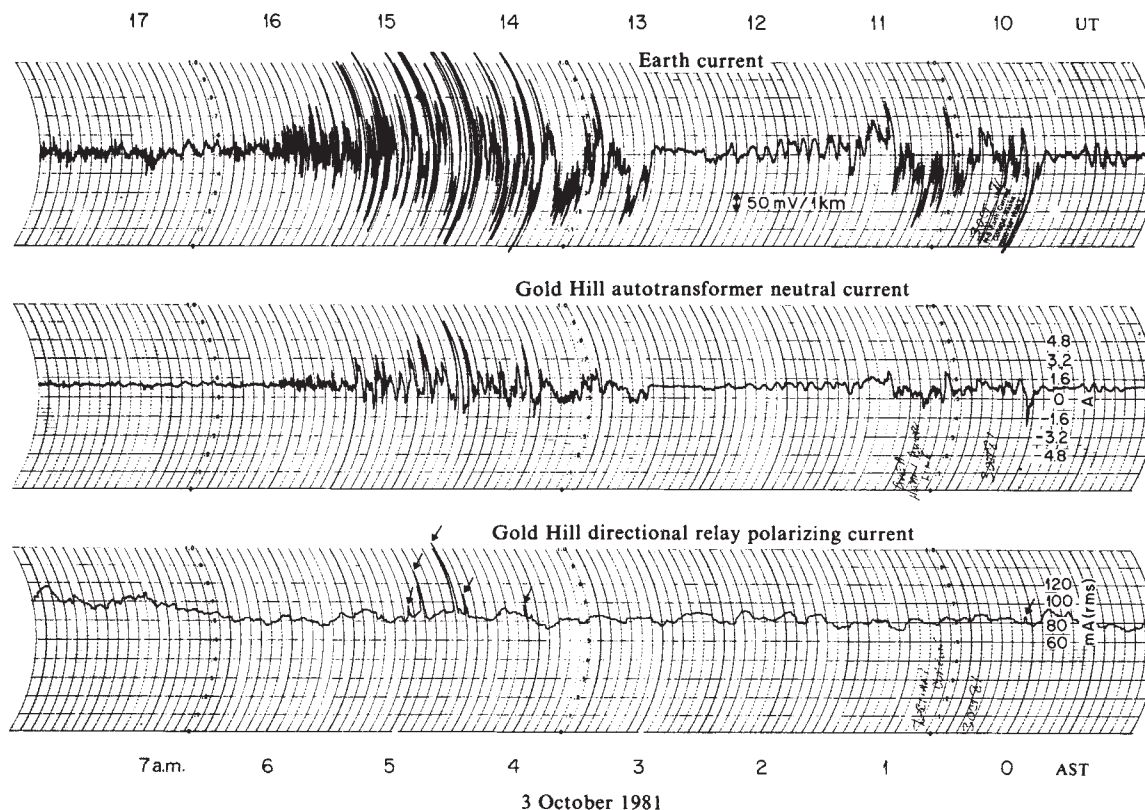


Fig. 1 Simultaneous recordings of Earth current (Earth surface potential), neutral current and protective relay system current (directional relay polarizing current) in the autotransformer located at the Gold Hill substation near Fairbanks on 3 October 1981. Excursions of $\pm 4 \text{ A}$ neutral currents represents $\pm 8 \text{ A}$ d.c. flowing in the transmission line. AST = Alaska standard time.

Fig. 2 Simultaneous recordings of aurora-induced surges in a protective relay system (directional relay polarizing current) and the neutral current in the Gold Hill autotransformer on 19 December 1980.

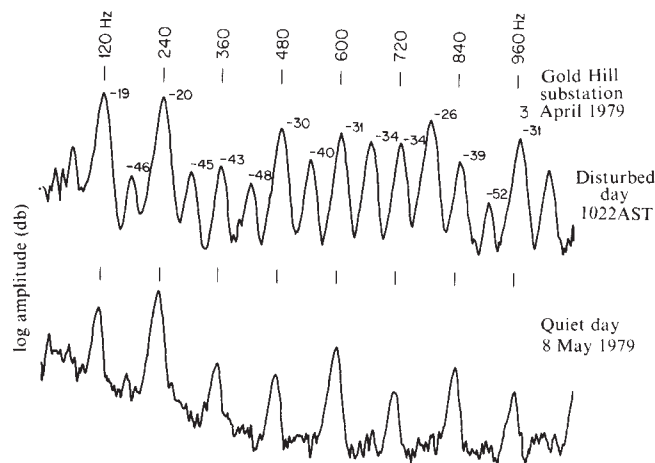
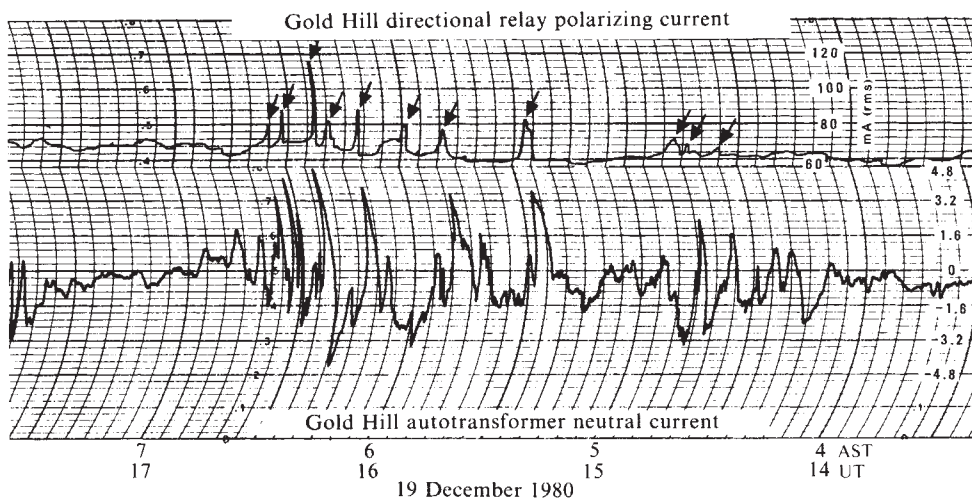


Fig. 3 Transformer sound spectra during a disturbed period (3 April 1979) and a quiet period (8 May 1979).

former⁸⁻¹⁰. Sound spectra were experimentally determined for the autotransformer for periods of both weak and substantial auroral activity at different occasions. This was achieved by a spectrum analyser processing tape-recorded sound measurements. Transformer sound occurs in 60 Hz power systems because ferromagnetic materials change size slightly when magnetized. This change in size (magnetostriction) normally occurs during each half cycle of the 60 Hz fundamental frequency or 120 times s^{-1} . As the magnetostriction process is nonlinear, all harmonics of 120 Hz are also present, contributing to transformer sound output. If d.c. excitation is present, however, the measured transformer sound spectrum contains the 60 Hz fundamental and all harmonics of 60 Hz. This occurs because magnetostriction is no longer symmetrical over successive half cycles of 60 Hz excitation. Figure 3 shows the sound spectrum for a disturbed period and a quiet period, respectively. Note that a large increase of all harmonics of 60 Hz would increase eddy currents in the transformer core and cause possibly destructive transformer overheating.

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Acid snow in the Canadian high Arctic

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Increasing levels of acid sulphates in precipitation have recently been discovered in locations as far north as Barrow, Alaska and Bear Island (74° N and 19° E) in the Norwegian Sea¹⁻⁴. The high sulphur concentrations are attributed to mid-latitude Eurasian sources⁵. We present here acid concentrations (each representing several years of snow accumulation) in ice from part of a 337-m surface-to-bedrock core on northern Ellesmere Island (81° N, 73° W) representing the past 5,000 yr and compare these with significantly higher acid concentrations in snow deposited over the past 25 yr at the same location showing seasonal variations in the concentrations which together constitute a significant trend of increasing acid levels over the past 25 yr.

Thermal core samples (Fig. 1) were taken in 1977 with a thermal drill at 1,600 m altitude within 1 km of the top of the Agassiz Ice Cap on northern Ellesmere Island, N.W.T., Canada (81° N 73° W). At this location melting occurs in most summers but affects only 3% of the surface annual layer. Samples from these cores were prepared and analysed in our Ottawa laboratory. The ice was first cleaned thoroughly with warm distilled, deionized and filtered water (10 MΩ). The sample was then allowed to melt partially; this melt was discarded. The final melt was collected for analysis and represented <50% of the original sample. The surface samples (Fig. 2) were collected in 1980 with a stainless-steel hand auger and split into two monthly increments. They were then stored in Whirlpak plastic bags that were free from contaminants. The samples were handled with plastic gloves cleaned by 'vigorous rinsing' in clean snow at the site. As these samples were considered uncontaminated for our purposes no cleaning procedures were used. Both core and surface samples were allowed to stand covered to reach room temperature before pH and conductivity (σ) measurements were taken on site. In addition to these samples 400 snow/firn samples were collected from a 10-m pit in 1977 at the same

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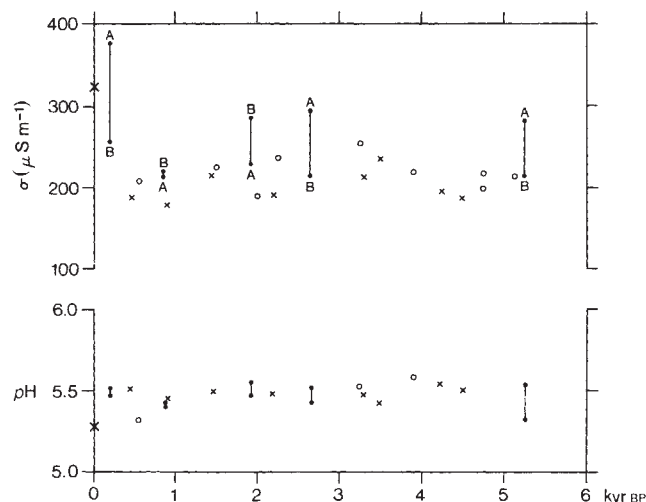


Fig. 1 Electrolytic conductivity (σ , measured with a Radiometer CDM 3m) and acidity (pH , measured on an Orion Research Ionanalyser) 0–5,000 yr BP. Agassiz Ice Cap, Canada. A and B represent periods before and after major volcanic eruptions that laid down acid layers on the ice cap. $\circ$, $\times$ mean values for samples consisting of five or more years of accumulation. The time scale is based on an analysis of seasonal variations of particulates (measured in a Coulter Counter) and K (measured by graphite furnace AA) at 15 levels in the core.

location. We also measured for Na, Mg, K and Mg using flameless atomic absorption and particulate concentrations.

The reproducibility of the trend of increasing σ with decreasing depth (Table 1) between two sets of samples collected quite differently is strong evidence of the absence of a sampling artefact in the results. However, the most serious 'contaminant' in our samples is CO_2 which appears as H_2CO_3 in our melted samples and increases the acidity. Water at $0^\circ C$ (sample just melted) contains about twice as much CO_2 as at the $20^\circ C$ at which we measured our samples. We did not ensure complete equilibration with ambient air and therefore some noise from a variable H_2CO_3 background may have affected our results. However, our samples were measured in random order and the trend we discuss later cannot have arisen in this way.

Snow/firn density increases with depth. Therefore, in any one sample the sample-to-air ratio also increases with depth. This lessens the equilibration with air of an enclosed sample with firn depth which, because of lower CO_2 loss from the sample with depth, should increase the acidity in the same direction. Our trend is precisely the opposite and must therefore be reduced by any CO_2 effect.

Our seasonal pH cycles agree well in shape and amplitude with the pH cycle in snow calculated from sulphate concentration in air over Mould Bay⁵ ($76^\circ N$ $120^\circ W$). The H^+ noise background due to H_2CO_3 is at most only about a sixth of the magnitude of our seasonal H^+ amplitude and a third of that of the total H^+ change over the past 27 yr and, as we have argued, does not contribute to it. While slightly <70% of the Holocene

acid (Fig. 1, Table 1) is calculated to be due to CO_2 -associated H^+ ion, the changes above this level, whether they are seasonal cycles or decadal trends, are not due to any variable CO_2 effect.

The Holocene results are shown in Fig. 1 (some results from our investigation of volcanic layers in the ice are included for comparison). The main feature of Fig. 1 is that the present-day (AD 1954–80) acidity ($pH = 5.23$) and σ ($311 \mu S m^{-1}$) show the snow is significantly more acid and conductive than snows from the preceding 5,000 yr. Only annual values at the peak of volcanic acid injections into the atmosphere (not shown in Fig. 1) exceed the present-day 25-yr pH value. We measured the core samples at our Ottawa laboratory which is some 1,500 m lower in altitude than the field camp where the surface samples were measured. Because of different levels of CO_2 in the air, this makes our Holocene value more acid than the true value (more H_2CO_3 in the sample melt). The 5,000 yr pH values for the Agassiz and Devon Island ice caps⁶ and Crête, Greenland⁷ show a Holocene background value equivalent to $\sim 1.0 \mu mol$ non- CO_2 associated H^+ ion kg^{-1} .

The seasonal variation in pH and conductivity over the 26-yr period which represents the 'present-day' mean is shown in Fig. 2. The acid and/or conductive peaks are at the surface in May and may be termed a spring peak, although concentrations show an irregular, but overall gradual, increase over the winter. Concentrations of Na, Ca, K, Mg and insoluble particulates are also at maximum in the spring. The ions tend to peak about a month before the insoluble particulates. We do not consider that spring peaking of various elemental constituents in the snow results from stratospheric injections due to tropopause folding mainly because the particulates increase by a factor of 10 whereas the acid concentration increases by a factor of three. Yet stratospheric chemistry shows a non-volcanic SO_4/Al ratio of 10:1 (ref. 8) (our pre-1963 values cover a volcanically quiet period). As most of the SO_4 ions are present as H_2SO_4 (ref. 8), our spring peak should show a 100-fold and not a 3-fold (4-fold in ref. 5) enhancement of the non- CO_2 associated H^+ ion if we have a 10-fold enhancement of Al (particulates). Over the past 26 yr (where our time scale is based on a knowledge of the surface accumulation rate and the seasonal pH and σ variations) the pH has shown an overall decrease (Fig. 3 and Table 1) of $0.007 yr^{-1}$ (or $0.007 \mu mol H^+ kg^{-1} yr^{-1}$). The pH and σ trends ($3.69 \mu S m^{-1} yr^{-1}$) are compatible with the increase in conductivity being due almost entirely to increasing levels of acid in the snow. If we project this trend backwards we find the acid began to increase from general Holocene values some time in the early 1930s. However, the overall trend may well not be a linear one.

We examine four possible explanations for this trend. First, increasing turbulence in the atmosphere over the past 26 yr might give rise to a longer transport of the H^+ ion without there being any increasing production rate. Second, there may have been decreasing levels of neutralizing components in the atmosphere. Third, there could have been a change in the precipitation or nucleating processes at the cloud level. Finally, we may be seeing a genuine increase in the levels of acid in the atmosphere, generally, or just along a preferred trajectory from a particular source.

Table 1 Particulate, acid, sodium concentrations and electrolytic conductivity of near surface snow and deep ice on Agassiz Ice Cap

Component	Concentration units	Holocene mean	Recent mean pit, 1950–77 (a) core, 1954–80 (b)	Recent trend per yr pit, 1950–77 (a) core, 1954–80 (b)
Particulates $d > 1.0 \mu m$	$No. \times 10^3 l^{-1}$	18.2	18.3 (a)	0.6 (a)
Na	p.p.b.	32*	25 (a)	0.47 (a)
Electrical conductivity	$\mu S m^{-1}$	201	314 (a) 311 (b)	3.3 (a) 3.7 (b)
Acid	pH	5.48	5.28 (a) 5.23 (b)	— –0.007 (b)

* All associated with volcanic episodes.

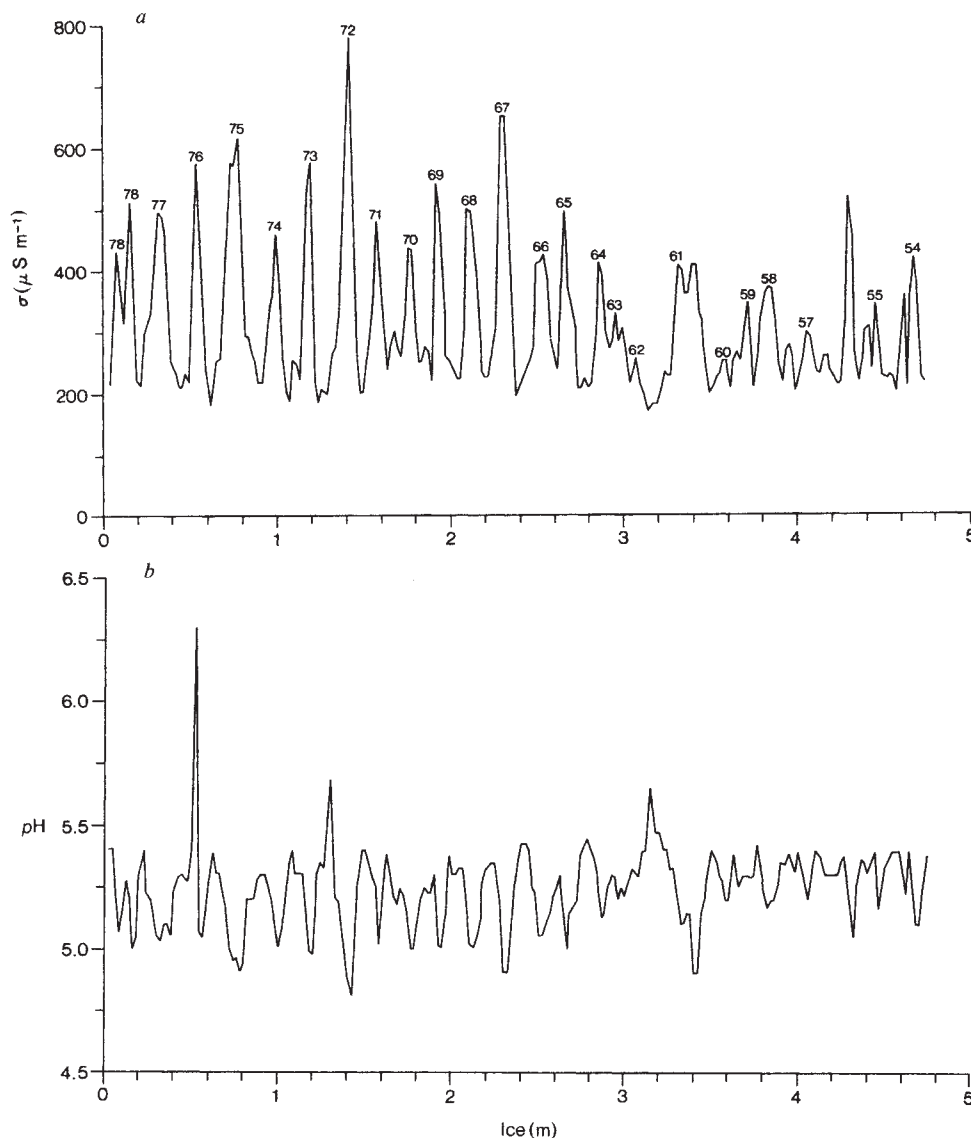


Fig. 2 Seasonal variations of *a*, electrolytic conductivity (σ), and *b*, acidity (pH) as measured on a 10 m core from Agassiz Ice Cap, Canada. The numbers refer to the spring in each year according to 72 = 1972.

From our pit results in 1977 we find significant trends of increasing concentrations of sodium and particulate matter in the snow (Table 1). At first sight this could be seen as evidence of increasing turbulence in the high Arctic over the same period. However, present-day particle concentrations (Table 1) are not significantly different to those over the past 5,000 yr. The same is true of our Devon Island core⁶ and also of the Crête core in Greenland⁷. This indicates that there have been no major changes in turbulence at the ice cap site or in the high Arctic generally over the same period. The lack of change in particulate concentrations would also suggest that there has been little general change in the neutralizing capacity of aerosols over the high Arctic in the past 5,000 yr.

Junge⁹ cautioned against considering that changes in concentrations of elemental components in ice cores represent or are the effect of similar changes in aerosol composition and concentration. He believes changes in the moisture content of precipitating clouds (*c*) and/or the mass fraction of aerosols used for condensation (ϵ_m) would cause changes in the concentration of the same aerosols when incorporated in the snow. However, we do not find a significant correlation between the changing acid concentrations and $\delta^{18}\text{O}$ in the snow. The $\delta^{18}\text{O}$ value is largely determined by the temperature of condensation at the cloud level¹⁰ and temperature at this level is probably the main determinant of *c* and possibly ϵ_m .

We are, therefore, left with the most likely explanation that increasing acid levels in the snow are due to similarly increasing

acid levels in the atmosphere over the ice cap and that this mutual increase is somehow allied to augmented acid production at its source. The source(s) could be natural (volcanic) or anthropogenic (industrial pollutants). There have not been steadily-increasing levels of volcanic sulphur injections into the atmosphere over the past two decades^{11,12} which does not favour a volcanic acid origin. Stratospheric SO_4 concentrations measured in quiet, that is non-volcanic, periods have been increasing by $9\% \text{ yr}^{-1}$ over the past 20 yr (refs 11, 12). The increase has been assigned an anthropogenic origin^{11,12}.

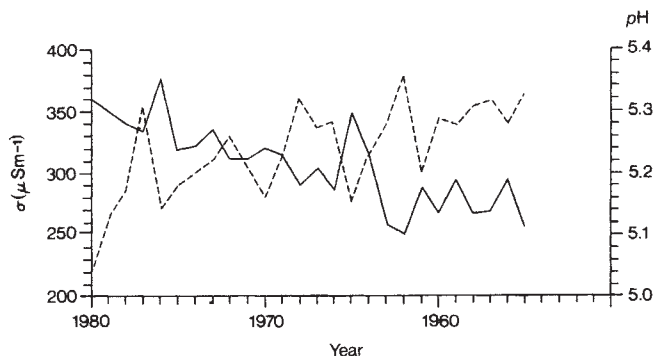


Fig. 3 Annual values (measured from spring to spring) of acidity (pH, dashed line) and electrolytic conductivity (σ , solid line) from a 10-m core on Agassiz Ice Cap, Canada.

However, because we do not detect the eruption of the volcano Gonung Agung in 1963 in our work (Fig. 2), we must be measuring a tropospheric acid input to the northern Ellesmere ice cap rather than a stratospheric one (which agrees with our earlier comments on the spring maximum of dust, acid and other ions). In the same tropospheric context high levels of industrial sulphates have already been shown for Barrow, Alaska^{1,2,4} Bear Island³ in the Norwegian Sea and Mould Bay in Arctic Canada⁵. Increases in sulphates have also been declared for Greenland over the past 20 or 30 yr (refs 13, 14) and for post-1970 snows compared with pre-1900 snow¹⁵. However, in recent work there is no evidence for a trend of increasing acid levels in the snow at Crête, Greenland⁷. Similarly, detailed electrolytic conductivity measurements made at the top of the Devon Island ice cap (1,800 m a.s.l.) and spanning more than 30 yr show no conductivity trend between 1942 and 1972. The lack of increasing conductivity in recent snows on the Devon Island Ice Cap, although the present-day value there is significantly higher than the Holocene one, may be due to the fact that the Devon Island Ice Cap has a very strong input of marine aerosols⁶ which

presents a strong noise background that is swamping the acid trend. However, we cannot ignore the possibility that the proposed acid trajectory across the Arctic Ocean from Eurasian sources^{1,2,4} excludes those high Arctic areas distant from the Arctic Ocean thereby favouring northern Ellesmere rather than Devon Island or Crête, Greenland. We should mention that preliminary results from a hand-cored profile at 82°N 77°W also on northern Ellesmere but closer to the Arctic Ocean than the Agassiz Ice Cap show an identical trend to the latter's of increasing acidity over the past 28 yr.

The full implications of increasing acid precipitation in the High Arctic are beyond the scope of this paper. However, the most acid snow is at the snow surface when melt begins in May or June in the high Arctic. Unless this melt refreezes at the base of the snowpack, the first runoff in spring will be the most acid. Acid levels in high Arctic snow, and their effects, should therefore be monitored on a routine basis as in certain areas, for example where lake fish form a source of Inuit income, there could be social implications.

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Molluscan biostratigraphy of the Koobi Fora hominid-bearing deposits

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Since the discovery¹ of late Cenozoic hominid-bearing deposits in the Koobi Fora area, north Kenya, a variety of studies^{2–9} have been undertaken to provide a chronostratigraphical and palaeoenvironmental context for the important palaeoanthropological and early archaeological discoveries^{1,10}. Unfortunately, although the exposures are areally extensive (>1,000 km²), they are broken up into subareas (Fig. 2); these local sections are often difficult to correlate with one another due to intervening bush cover and faulting. Here I summarize a molluscan biostratigraphical analysis which demonstrates serious stratigraphical miscorrelations between certain local sections, including some previously indicated by vertebrate evidence. These miscorrelations require substantial revisions to current stratigraphical interpretation of the Koobi Fora deposits. In particular, both the Suregei and Tulu Bor tuff horizons actually represent different levels. Although the Tulu Bor is considered to overlie the Suregei, some exposures currently attributed to the Tulu Bor are actually older than some units previously mapped as the Suregei. Fortunately, the stratigraphical miscorrelations reported here do not significantly affect the relative placement of the mollusc faunas reported in a previous evolutionary analysis¹⁵; superpositional relationships in the areas from which these faunas were collected (mainly collecting areas 102, 103, 110, 123, 202 and 203) are unaffected by the miscorrelations reported here.

Lithostratigraphical correlations in the Koobi Fora area have been based on certain tuffaceous horizons which regularly punctuate the late Cenozoic sequence. These tuffs are generally secondarily waterlain^{5,6}; their bases have been considered laterally extensive, mappable and essentially isochronous^{11,12}. The supposed recognition of these tuffs in geographically-

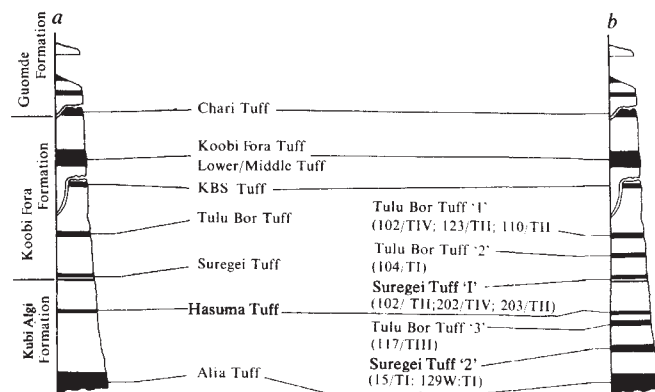


Fig. 1 Stratigraphical relationships of late Cenozoic deposits of the Koobi Fora region. A, Currently accepted stratigraphical framework (after ref. 7). B, Generalized revisions suggested by this report. Note that several tuffs previously allocated to the Suregei and Tulu Bor Tuffs actually represent horizons of widely differing age. These various horizons are designated by the informal terms 'Tulu Bor '1'', 'Tulu Bor '2'', and so on. This terminology, adopted here solely to indicate which horizons were previously considered lateral correlates, has no formal status whatsoever. The different Tuff horizons previously allocated to the Suregei and Tulu Bor Tuff levels are also distinguished by the 'area/succession' scheme suggested by Harris and White¹³; a given tuff exposure is designated by the collecting area in which it occurs, and by its stratigraphical position with respect to other tuffs within the local section of that collecting area. Thus, the designation 102/TI indicates the earliest (lowest) tuff in the local stratigraphical sequence in collecting area 102. Total thickness of the Kubi Algi, Koobi Fora and Guomde Formations is considered to be ~350 m (ref. 7).

separated local sections has formed the basis of a regional chronostratigraphical scheme^{5–7} (Fig. 1a); this scheme has provided a framework for palaeontological discoveries and palaeoenvironmental interpretations. The validity of this framework, and of all interpretations based on it, relies entirely on the correct recognition and correlation of the various tuffs in widely separated local sections. But these tuffs are laterally variable in field aspect, and proposed correlations between local sections are therefore often questionable. In addition, vertebrate studies have suggested miscorrelations between certain tuffs^{13,14}.

The profound evolutionary changes recorded in the mollusc faunas of the Koobi Fora area¹⁵ permit the recognition of several events which are time-significant for the basin. Putative correlations between exposures of marker tuffs in various areas were therefore tested using these faunas. Molluscan biostratigraphical type sections were designated in the same general areas as the lithostratigraphical type sections for the three late Cenozoic formations of the area, and on the basis of the evolutionary events recorded in 40 mollusc faunas from these type sections, 10 concurrent range zones were defined (Fig. 3); 112 faunas collected in other areas, away from the type sections, were then allocated to the appropriate biozone. When these latter faunas occur close to local exposures of the various marker tuffs, the position of these tuff exposures relative to the molluscan type section can be more or less accurately determined.

Although the biostratigraphical type sections for each of the three late Cenozoic formations in the area are continuous local sections, these sections are, like the lithostratigraphical type sections, geographically separated (Fig. 2). However, the superpositional relationships of these sections are unambiguous¹⁶. Together, these three sections exhibit 9 of the 10 proposed molluscan biozones (zones 2–10). Zone 1 is not certainly developed in the lithostratigraphic type section of the area; the type section for this zone is the Karsa Waterhole sequence¹⁷, which appears either immediately to predate the Kubi Algi Formation, or to correspond to some level in the lower half of this unit (I.C. Findlater, personal communication) (no mollusc faunas are known from the lower half of the type section of the Kubi Algi).

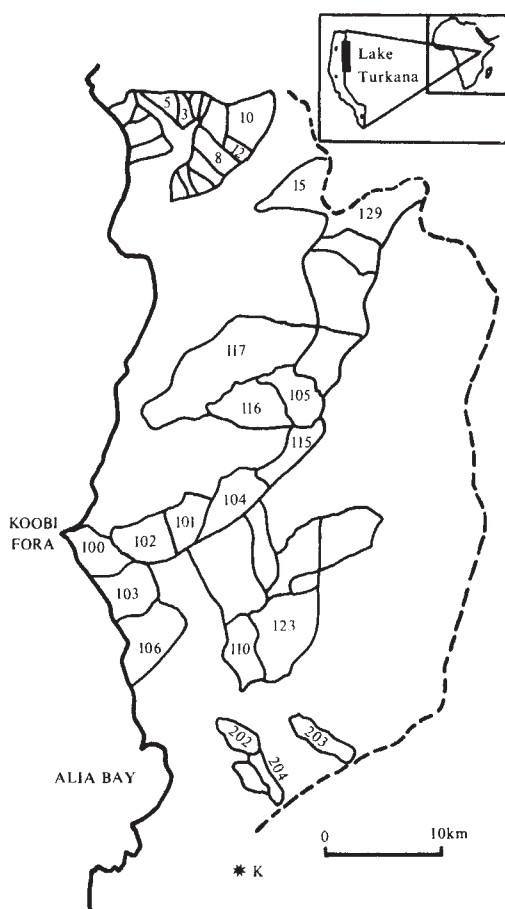


Fig. 2 Principal palaeontological collecting areas designated in the Koobi Fora area, north Kenya. Molluscan biostratigraphical type section for the Kubi Algi Formation is in area 203, that for the Koobi Fora Formation in area 102, and that for the Guomde Formation in area 3. These type sections encompass molluscan biozones 2–10; the type section for zone 1 is the Karsa Waterhole sequence¹⁷ ('K'), to the south of the basin margin (indicated by heavy broken line).

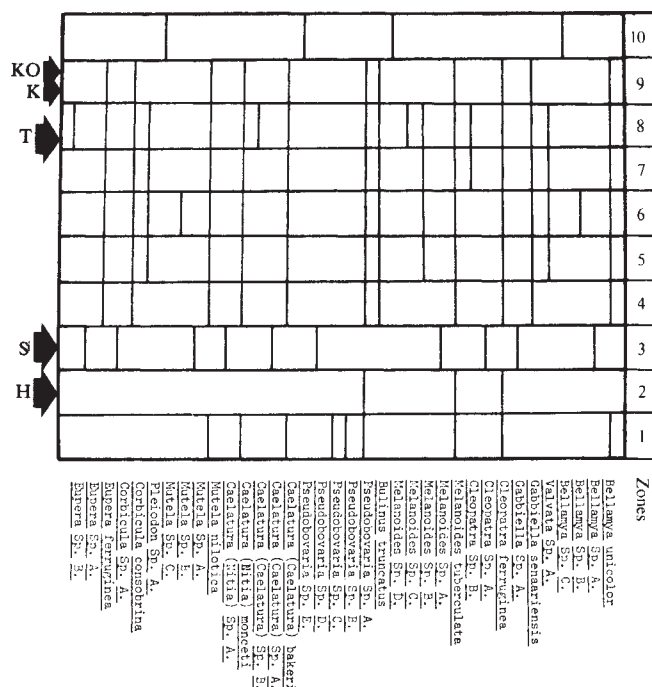


Fig. 3 Range zones of freshwater molluscs from the Plio-Pleistocene deposits of the Koobi Fora area. Zone 1 faunas are characterized by the presence of two distinctive forms of *Pseudobovaria* and by the absence of *Corbicula*. Zone 2 faunas consist of widely distributed and generally still extant taxa, but are taxonomically restricted and dwarfed, reflecting the period of high alkalinity consequent on the marked regressive phase at and above the Hasuma Tuff level⁷. Zone 3 faunas document contemporaneous speciation events in all lineages in the basin during a continuation of this regressive phase, and presumed geographical isolation of faunas¹⁵. Zone 4 faunas document the elimination of the zone 3 endemics as typical ancestral stocks return to the basin, coincident with a major transgressive phase. Zones 5–8 document a progressive intrabasinal radiation of several of these stocks and the accumulation of certain 'exotic' taxa in the Lower Member of the Koobi Fora Formation. Zone 9 documents a major reduction in faunal diversity; all the endemics and exotics of zones 5–8 were extirpated by the rise in alkalinity²⁴ in the uppermost Lower Member consequent on a climatically induced²⁵ regression^{5,26}. The zone 10 faunas from the Guomde Formation document a second series of contemporaneous speciation events in the various lineages present in the basin, again coincident with major regression and presumed geographical isolation. Widths of zones as drawn do not reflect their relative stratigraphical thicknesses. Heavy arrows to side of diagram reflect the placement of certain tuff horizons with respect to the molluscan biozones, in the biostratigraphical type section. H, Hasuma Tuff; S, Suregei Tuff; T, Tulu Bor Tuff; K, KBS Tuff; KO, Koobi Fora Tuff.

The evolutionary events previously documented¹⁵ in the mollusc faunas of the Koobi Fora region actually form the basis for the differentiation of zones 2–10 inclusive. The faunas here attributed to zone 1 have not been reported previously; they are characterized by the presence of two distinctive species of *Pseudobovaria* and by the absence of the now extremely widespread and eurytopic genus *Corbicula*. One or other of these forms of *Pseudobovaria* have been previously recognized by Vandamme from either the Kanapoi deposits to the south of the Turkana Basin, or from the lower parts of the Omo Valley sequence to the north¹⁸. Both forms of *Pseudobovaria* apparently became extinct during the high alkalinity phase documented by the stunted zone 2 faunas; *Corbicula* appears to have been a rather recent Eurasian immigrant into Africa¹⁹. Zone 1 faunas are also now known from the Warata Formation²⁰, north-east of the Koobi Fora area.

The stratigraphical positions of various marker tuff horizons relative to the biostratigraphical type section can be determined when the mollusc faunas bracketing them are allocated to the proposed biozones. Clearly, the precision with which a tuff horizon in a given local section can be related to the biostratigraphical type section will be determined by how closely it is bracketed by zonally attributable mollusc faunas. The relative

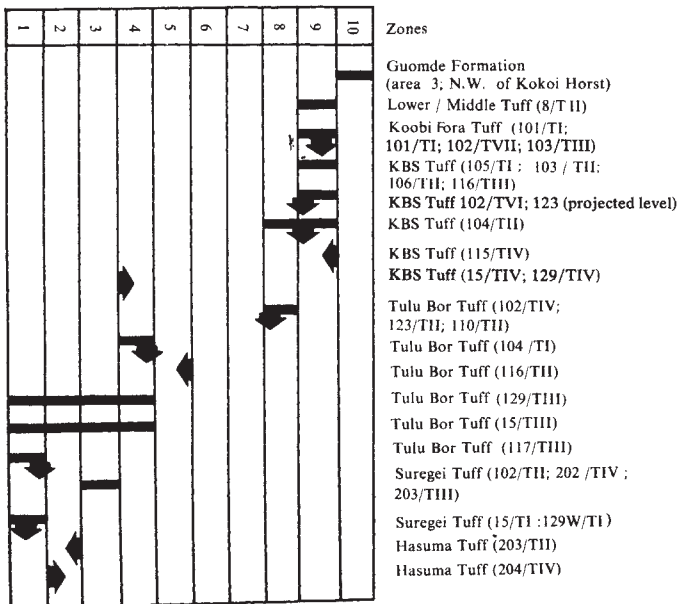


Fig. 4 Positions of various exposures of the major marker tuffs, and of Guomde age sediments, with respect to the various molluscan biozones. Possible range of positions of the tuffs within the zonal scheme indicated by heavy vertical bars. Most probably positions of the various tuffs, suggested by superpositional data and by propinquity to zone boundaries, indicated by horizontal arrows. Upward and downward facing arrows indicate the uppermost and lowermost possible positions, respectively, of poorly bracketed tuffs. Note that horizons attributed to both the Tulu Bor and Suregei levels fall into a wide range of biozones, suggesting the stratigraphical revisions indicated in Fig. 1b. Notations after tuff names indicate the geographical and stratigraphical positions of the various tuff horizons according to the scheme suggested by Harris and White¹³; a given tuff exposure is designated by the collecting area in which it occurs, and by its stratigraphical position with respect to other tuffs within the local section of that collecting area. Thus, the designation 102/TI indicates the earliest tuff in the local stratigraphic sequence in collecting area 102. Allocation of the horizon attributed to the Suregei Tuff in areas 15 and 129 to zone 1 assumes the apparently firm short-distance lithostratigraphical correlation between these areas to be correct (this horizon is immediately underlain by zone 1 faunas in area 15, and immediately overlain by zone 1 faunas in area 129).

positions of several important exposures of marker tuffs, determined as accurately as possible by this method, are indicated in Fig. 4. Several serious miscorrelations of tuffs have occurred. In particular, various horizons allocated to the important Suregei and Tulu Bor Tuffs fall, in both cases, into different biozones; both stratigraphical terms therefore encompass several horizons of differing age. (Studies on fossil suids from these deposits have previously suggested that certain exposures assigned to the Tulu Bor Tuff in fact pre-date the Suregei Tuff horizon of the type section of the Koobi Fora and Kubi Algi Formations^{13,14}). A generalized and tentative revised stratigraphy suggested by this analysis is indicated in Fig. 1b. Only tuffs whose relative positions with respect to the biostratigraphical type section are unambivalent are indicated; the remaining, less securely bracketed tuffs shown in Fig. 4 may or may not be lateral correlates of those shown in Fig. 1b. In addition, due to the absence of faunal evidence from the Alia Tuff, its stratigraphical position with respect to the 'Tulu Bor 3' and 'Suregei 2' horizons (see Fig. 1) is unclear.

The present study has three immediate implications:

(1) The Plio-Pleistocene deposits of the Koobi Fora area were originally divided by Bowen and Vondra²¹ into two main stratigraphical units, the Kubi Algi and overlying Koobi Fora Formations and the boundary between them was formally defined as the base of the Suregei Tuff²¹. The type locality of the Suregei Tuff lies close to the Suregei escarpment (from which it is named) in area 15 (refs 13, 14) (see Fig. 2). Unfortunately, as demonstrated above (Fig. 4), this tuff exposure is substantially older than its supposed correlate horizon in areas 102, 202 and

203 (Koobi Fora-Alia Bay area). But this latter horizon lies within the formal type sections of both the Kubi Algi and Koobi Fora Formations! Clearly, the terms 'Kubi Algi Formation', 'Koobi Fora Formation' and 'Suregei Tuff' must be either redefined or abandoned.

(2) In terms of the currently accepted stratigraphical scheme, large areas of exposure in areas 117, 129 and 15, previously allocated to the Koobi Fora Formation, are considerably older than the lithostratigraphical type section of this unit, and apparently correspond in age to some part of the type section of the Kubi Algi (see also refs 13, 14).

(3) Hitherto, the apparent restriction of Kubi Algi age sedimentation to the southern part of the Koobi Fora region (Alia Bay area) implied a generally regressive regime in the earlier Plio-Pleistocene⁷, succeeded by a major transgression at the (apparently) areally extensive Suregei Tuff level⁷. In contrast, the Mursi, Usno and Lower Shungura Formations in the neighbouring Omo area document intermittently widespread lacustrine conditions throughout this period^{22,23}. However, recognition that Kubi Algi age sedimentation occurs throughout the Koobi Fora area, and that the 'Suregei Tuff' level consists of at least two separate horizons and does not represent one extensive depositional event during a single major transgression, implies a similar picture of intermittent lacustrine conditions in the earlier Plio-Pleistocene throughout the Koobi Fora region. These observations bring the depositional histories of these two regions into greater congruence, suggesting that widespread lacustrine conditions pertained intermittently throughout the Turkana Basin in the earlier part of the Plio-Pleistocene depositional cycle.

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Aneural muscle cell cultures make synaptic basal lamina components

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The basal lamina that runs between the presynaptic and post-synaptic membranes at the neuromuscular junction has distinctive functional properties¹⁻³ and differs in molecular composition from the extrasynaptic basal lamina to which it is attached^{4,5}. In regenerating muscle, synaptic basal lamina can direct the accumulation both of neuronal transmitter vesicles at specific sites near the presynaptic nerve terminal membrane and of acetylcholine receptors (AChRs) in the postsynaptic muscle membrane¹⁻³. Four components that are concentrated in synaptic basal lamina, but are not detectable in extrasynaptic basal lamina, have been recognized. The first is a particular form of acetylcholinesterase (AChE), the A₁₂ or 16S species, which is concentrated at the synapse in rats^{6,7} and mice⁸; its distinctive feature is thought to be a long collagen-like tail⁹ that attaches it to the basal lamina¹⁰. Three other components of the synaptic basal lamina have been distinguished immunocytochemically using antisera raised against basement membrane extracts or collagen-rich preparations⁴. Although it has been previously reported that aneural muscle cell cultures may make the 16S form of AChE^{11,12}, the role of the nerve in the synthesis and organization of the synaptic basal lamina is unknown. We report here that cells of a mouse muscle line, in the absence of nerves, have surface accumulations of each of the four synaptic basal lamina components described above: the 16S AChE, and cross-reactive antigens recognized by each of the synapse-specific antisera.

Cells of the C₂ line, derived from regenerating skeletal muscle of adult C3H mice¹³, proliferate as myoblasts in high serum medium (Dulbecco's minimal essential (DME) medium containing 0.5% chick embryo extract and 20% fetal calf serum) and fuse into myotubes in low serum medium (DME with 10% horse serum). Within 3 days after the medium is changed, the cultures contain numerous long, cylindrical, multi-nucleated myotubes that contract spontaneously. The synapse-specific basal lamina antigens were detected using three sera described by Sanes and Hall⁴: we refer to the serum produced against solubilized anterior lens capsule as 'junctional-specific I' (JS-I); to the serum raised against muscle basement membrane collagen, and subsequently adsorbed with extrasynaptic muscle basement membrane so as to reveal synapse-specific antigens, as JS-II; and to the serum produced against lens capsule collagen and similarly adsorbed, as JS-III. Some experiments were also performed with the unadsorbed sera, which stain both synaptic and extrasynaptic basal lamina. Immunofluorescence observations were made as described by Sanes and Hall⁴. Methods for detection of AChE are described in detail elsewhere^{8,14,15}.

C₂ myotubes, cultured without nerve cells, synthesized the synapse-specific A₁₂ form of AChE. After 5 days in fusion medium, ~30% of the total AChE activity sedimented at 16S (Fig. 1a). Histochemical examination of these fibres showed a patchy distribution of enzyme activity on the myotube surface (Fig. 1b). Such focal accumulations of AChE have been observed by others in primary myotube cultures from rat or chick embryonic myoblasts only in the presence of nerves^{12,16-18}. Focal accumulations of AChE in such nerve-muscle co-cultures appeared at the same time as the A₁₂ form of the enzyme^{12,18}. We have demonstrated, and report in detail elsewhere, that the

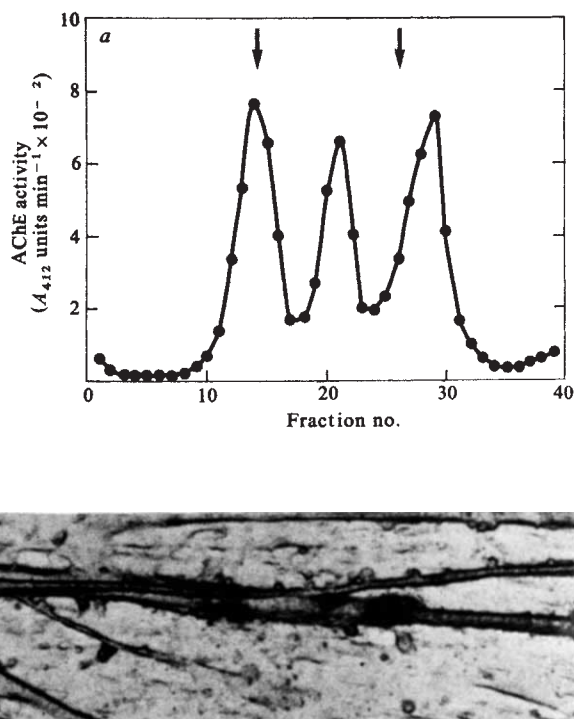


Fig. 1 Molecular forms and focal accumulations of AChE in C₂ myotube cultures. C₂ myotubes were grown for 5 days in fusion medium on a tissue culture plastic substrate and then examined for esterase activity. *a*, AChE was extracted from myotubes in a high ionic strength (1 M NaCl) buffer containing 0.5% Triton X-100, 20 mM EDTA and protease inhibitors⁸, and analysed by zone sedimentation on a sucrose gradient⁶. Enzyme activity was measured by the Ellman procedure¹⁴. The arrows indicate the position of (left) β -galactosidase (16S) and (right) alkaline phosphatase (6.1S). *b*, Esterase activity was localized *in situ* using the histochemical method of Karnovsky and Roots¹⁵ following fixation with 1% paraformaldehyde and 0.8% glutaraldehyde in 0.13 M sucrose buffered with 90 mM sodium phosphate, pH 7.4 (ref. 17). $\times 335$.

patches of AChE observed in aneural C₂ cultures correspond to the A₁₂ form of enzyme and are apparently associated with the extracellular matrix^{8,19}.

To visualize the extracellular matrix produced by C₂ myotubes, we used antisera that bind uniformly to synaptic and extrasynaptic basal lamina in rat muscle⁴ and mouse muscle (L.S. and Z.W.H., unpublished) (see Fig. 2a). The matrix formed a network of fibrils that stretched from cell to cell connecting the unfused myoblasts and myotubes, while an uneven pattern of staining outlined each myotube. Antibodies bound at low density over large areas of the myotube surface; more intense staining was seen in small patches and in long, thin arcs. None of these patterns was observed in control cultures that had been incubated with non-immune serum. These results show that the C₂ cells produce an extensive extracellular matrix.

We observed a more restricted distribution of immunofluorescence following incubation of the cultures with the synapse-specific antisera. In most cases, the antigens appeared as either arcs (Fig. 2c) or patches (Fig. 2b,d) on the myotube surface. Myotubes stained with the JS-III serum often displayed areas of fluorescent speckles (see Fig. 2d), a pattern not seen with the other synapse-specific antisera. Adsorption of each of the synapse-specific sera with C₂ myotubes abolished the staining of endplates in frozen sections of mouse muscle. Thus, the antibodies bound by C₂ myotubes are those recognized by the adult muscle synaptic basal lamina.

The arcs and small patches resembled the distribution of clusters of AChRs seen in separate experiments. Simultaneous labelling of the receptors with rhodamine-conjugated α -bungarotoxin and of the antigens with fluorescein-conjugated

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antibody demonstrated that in many cases the antigens were restricted to areas of the myotube surface containing high concentrations of AChRs. As shown in Fig. 3, the coincidence of the two was often striking. The greatest coincidence of antigens with AChR clusters was observed with the JS-I serum. In cultures examined 3 days after fusion, 30–45% of all receptor clusters coincided with patches of JS-I antigen(s). Similarly, the JS-II antigen(s) sometimes appeared in patches whose distribution was identical to that of AChR clusters. In both cases, however, antigen patches unrelated to receptor clusters were also present. Co-localization of the JS-III antigen(s) with AChR clusters has thus far not been observed in C_2 myotube cultures. Because at least one synaptic basal lamina component, JS-III, seems to be unrelated to receptor clusters whereas the other two sometimes are, the distributions of each of the synaptic basal lamina antigens cannot be completely coincident.

The presence of synaptic basal lamina components in aneural C_2 myotube cultures can perhaps be understood in the context of myoblast maturation. Several studies suggest that the myoblast's potential for gene expression changes through a maturation sequence that is dependent on innervation. For example, Koenig and Vigny¹² observed that myotubes in primary rat muscle cultures could make 16S AChE in the absence of nerves only if derived from embryonic muscles that had already been innervated; myotubes formed by cells from uninnervated muscles could make 16S AChE only if co-cultured with nerves. Similarly, in chick muscle, both the ability of myoblasts to fuse *in vitro* and the morphology of myotubes seem to be regulated by

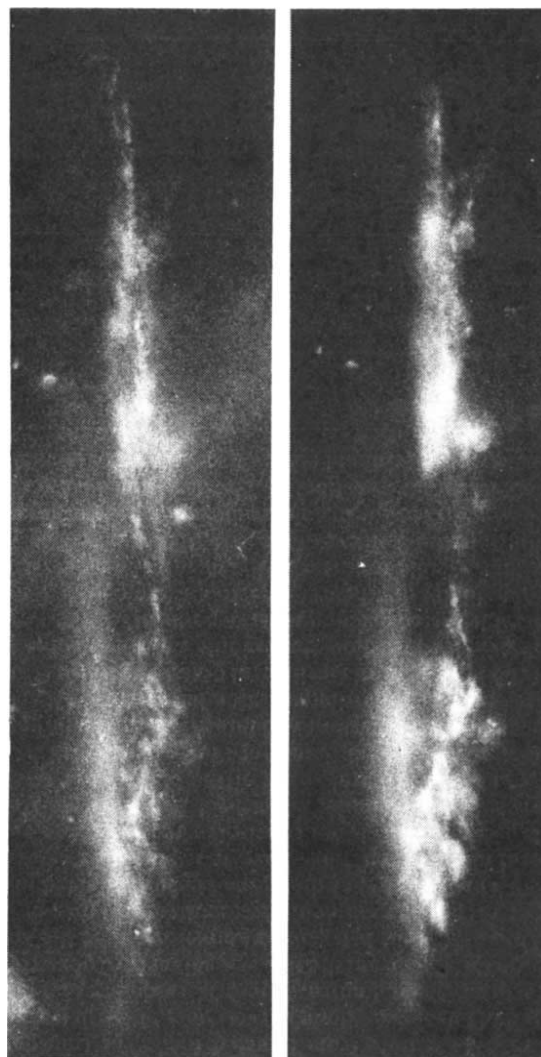


Fig. 3 Simultaneous localization of AChR clusters and basal lamina antigens in C_2 myotube cultures. Cells were plated on plastic coverslips, grown for 5 days in fusion medium and incubated with fluorescent reagents as before. Left, localization of JS-I antigen(s) by labelling with fluorescein-conjugated goat anti-rabbit serum as before. Right, AChR cluster in the same field observed after incubation with 50 nM rhodamine-conjugated α -bungarotoxin. Receptor clusters in control cultures, incubated with non-immune serum and photographed and printed in identical conditions, could be seen with rhodamine, but not fluorescein, filters. No receptor clusters were seen in cultures preincubated with unlabelled α -bungarotoxin (1 μ M), followed by rhodamine-conjugated α -bungarotoxin. $\times 1,564$.

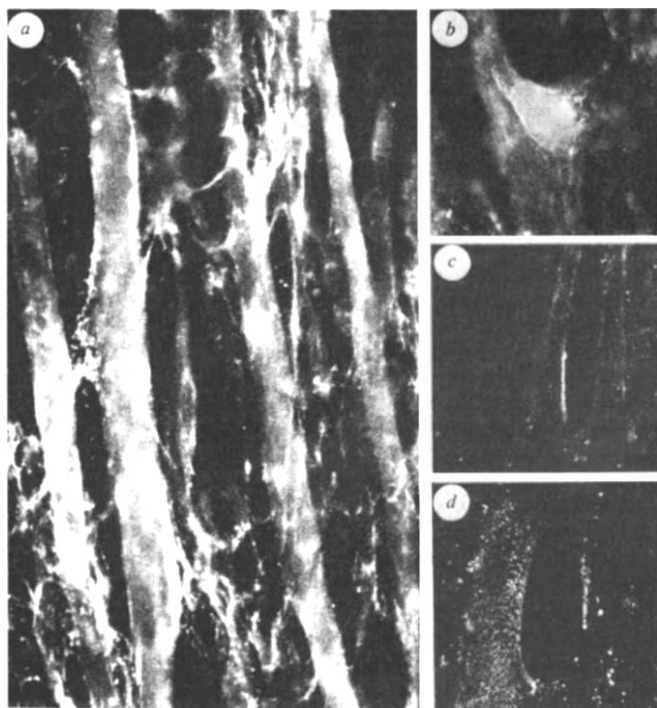


Fig. 2 Fluorescent antibody localization of extracellular matrix in C_2 myotube cultures. Cells were plated on tissue culture plastic and grown for 5 days in fusion medium, then incubated with rabbit antisera directed against the muscle basal lamina⁴ at 1/100 dilution in low serum medium (1 h, 37 °C), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit serum (Cappel) at 1/100 dilution in medium (1 h, 37 °C), washed with 0.15 M NaCl, 20 mM sodium phosphate, pH 7.2 (PBS) in 1 mM $CaCl_2$, mounted under 10% Gelvatol 20/30 (Monsanto), 20% glycerol in PBS, and examined with a Leitz Ortholux II microscope equipped with L2 (excitation bandpass (BP) 450–490, barrier BP 525/20) and N2 (excitation BP 530–560, barrier BP 580) filters. Reaction with: a, anti-muscle basement membrane collagen⁴ (similar results were observed after incubation with anti-lens capsule collagen, data not shown); b, JS-I; c, JS-II; d, JS-III. $\times 236$.

neural contact within the limb bud^{20–25}. The ability of C_2 myotubes to express 16S AChE, as well as other synapse-specific components of the basal lamina, is consistent with this scheme, as the cell line is derived from adult muscle; that is, the C_2 cell line may represent an advanced stage of myoblast differentiation that results from *in vivo* exposure to neuronal influences before isolation of the myoblasts.

In this context, the presence of synapse-specific basal lamina components in aneural C_2 muscle cultures does not imply that nerves are without influence on the normal appearance or localization of synaptic basal lamina components. We have shown previously that nerves can induce the appearance of synapse-specific basal lamina at sites of new endplate formation in adult muscle²⁶. It will be of interest to investigate the role of nerves in the organization and production of synaptic basal lamina in myoblast cultures of different developmental ages.

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Hypothalamic paraventricular nucleus is a privileged site for brain–pituitary interaction in long-term tissue culture

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It is generally accepted that hypothalamic factors are released from the median eminence into the hypophyseal portal vessels and exert a powerful control on the secretion of anterior pituitary hormones¹. The brain sites involved in the synthesis of hypothalamic factors—particularly those that control ACTH secretion^{2,3}—are, however, not yet established. It is also not known whether these sites are targets for pituitary hormone action, although recent anatomical⁴ and physiological studies^{5,6} suggest that pituitary hormones may reach the brain. For these reasons, we co-cultured^{7,8} various hypothalamic explants from rat brain for 4 weeks with anterior pituitary. We report that during the third and fourth week *in vitro*, there is an approximately 10-fold increase in bioactive ACTH^{9,10} in the medium of co-cultures prepared from the paraventricular nucleus and anterior pituitary, and that this increase is highly correlated with an increase (up to 10-fold) in concentrations of immunoreactive^{11,12} arginine vasopressin (AVP). Hormone levels did not increase when basal hypothalamus was co-cultured with anterior pituitary, paraventricular nucleus with posterior pituitary, or supraoptic nucleus with anterior pituitary. Thus, we propose a specific interaction between anterior pituitary and paraventricular nucleus that results in a mutual enhancement of each other's peptide production.

The hypothalamus of Sprague–Dawley rats aged 6 to 7 days of either sex was cut within the frontal plane into slices about 400 μ m thick, starting at the level of the optic chiasm (slice 1) and ending at the level of the posterior median eminence (slice

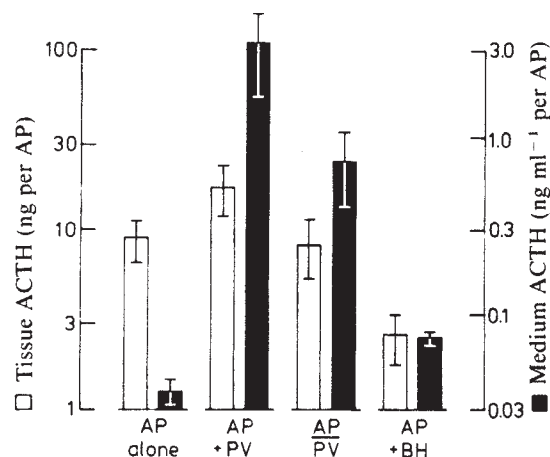


Fig. 1 Bioactive ACTH content (mean $\pm$ s.e.m.) of tissue (open bars) and medium (solid bars) from five to six hypothalamic–anterior pituitary cultures (AP, anterior pituitary; PV, paraventricular nucleus; BH, basal hypothalamus). AP + PV denotes a co-culture of the two explants in close apposition, as shown by the example of a AP + SO co-culture in Fig. 4a. In contradistinction, AP/PV signifies that the two explants were cultured on separate opposing coverslips within the same culture tube. The explants were embedded in a plasma clot and cultured in Falcon roller tubes. The medium (volume 1 ml) contained 25% horse serum, 25% Hanks' balanced salt solution, 50% basal medium (Eagle) and 6.5 mg ml⁻¹ glucose^{7,8}. The medium was replaced every week; hormone levels of the fourth week medium only are shown above. The tissue was homogenized in 700 μ l of 0.2 M acetic acid and centrifuged at 15,000 r.p.m. and 4 °C for 15 min. The supernatant and the pellet, resuspended in 0.5 ml of 0.2 M NaOH, were frozen at –20 °C pending assays of hormones and proteins. Bioactive ACTH content of the tissues and media was measured by adding 5 or 10 μ l of the 700 μ l supernatant and 100 μ l of the AP culture medium, respectively, to a 1 ml suspension of adrenocortical cells and by measuring the corticosterone secretion^{9,10}. Note log display in this and all other figures.

4). The paraventricular (PV) and supraoptic (SO) nuclei were punched out with a needle of 0.75-mm diameter by the Palkovits method¹³ from slice number 1, and the basal hypothalamus (BH) from slice number 3 and 4. The tissues were cultured as explants^{7,8} either alone or side by side with one half of an anterior pituitary (AP). Three half APs (three for technical reasons) were cultured alone as a control. The medium was collected and replaced every 7 days. After 28 days, the cultures were inspected by phase-contrast microscopy, and only those paraventricular and supraoptic explant combinations that displayed large size neurones were processed. The tissues were extracted in 0.1 M HCL, and the supernatants and pellets were stored at –20 °C pending hormone^{9–12} and protein¹⁴ assays.

When PV was co-cultured with AP for 28 days, the ACTH content of the medium (Fig. 1, solid bars, AP + PV) was 30 to 50 times larger than that of APs cultured alone or together with BH ($P < 0.025$). This large amount of medium ACTH was not due to a loss of ACTH from the tissue, since the tissue ACTH content (Fig. 1, open bars, AP + PV) was at least twice ($P < 0.05$) that found in any other type of cultures (AP alone, AP + BH). To test whether direct contact between the explants is necessary (see Fig. 4a), PV and AP were also cultured on separate opposing coverslips within the same tube. The ACTH content of the medium (Fig. 1, PV/AP) was at least 10 times larger than that of AP cultured alone, demonstrating that a factor released into the medium probably is responsible for the large increase in ACTH secretion induced by co-cultured paraventricular tissue.

The AVP contents of tissue (Fig. 2, open bars) and medium (dark columns) were about six times larger in PV–AP co-cultures than in PV cultures ($P < 0.05$). A trend towards elevated AVP levels was also observed when PV and AP were cultured on separate coverslips (AP/PV). Plotting the medium AVP and ACTH as a function of time in culture (Fig. 3) revealed a remarkable coincidence between the changes of both hormone

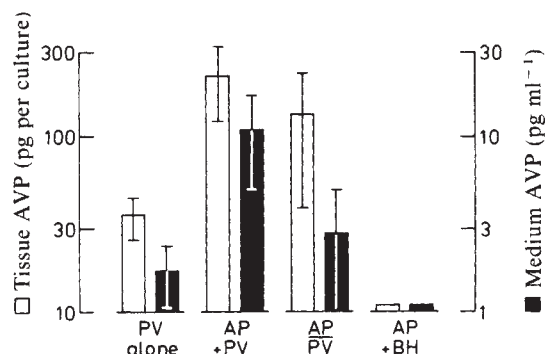


Fig. 2 Immunoreactive arginine vasopressin (AVP) content (mean $\pm$ s.e.m.) of tissue (open bars) and medium (solid bars) of the cultures shown in Fig. 1. For abbreviations, see Fig. 1 legend. The AVP content of the tissue was measured without extraction and the AVP content of the medium following vycor extraction by a specific radioimmunoassay^{12,13}. AVP in the samples dilutes according to standard curve. The AVP tracer was from NEN, the AVP standard (500 IU mg⁻¹) is a gift of Drs K. Lederis and R. Mathison. The anti-vasopressin antibody, a gift of Dr J. Durr, does not cross-react with oxytocin, DD-AVP, ACTH, α -MSH, β -endorphin or with the enkephalins. The antibody was used at a dilution of 1:200,000. Recoveries were controlled in assay buffer and culture medium and varied from 58% to 70% between assays and $\pm 3.5\%$ within assays. Sensitivity was 0.25 pg per tube, about 0.4 pg per ml medium and 10 pg per culture. AVP was not detectable in AP+BH or BH alone. In AP/PV cultures where the two explants could be extracted separately, only the PV extract contained AVP. Separate experiments (not shown) indicate that co-cultures of PV with either posterior pituitary, hippocampus or cerebellar explants have AVP levels similar to those of PV alone.

levels which is also illustrated by a strong correlation between $\log(\text{ACTH})$ and $\log(\text{AVP})$ ($=0.91 \pm 0.04$).

In view of the possible role of AVP as a corticotropin releasing factor (CRF) *in vivo* and *in vitro*^{10,15,16}, we investigated whether the presence of vasopressin or vasopressinergic cells was sufficient to stimulate ACTH release from the anterior pituitary. We therefore added 100 pg of synthetic AVP (Ferring) once every week to anterior pituitaries. Medium ACTH levels were about 30% higher than controls ($P < 0.05$) and AVP levels ranged from 2–20 pg ml⁻¹, demonstrating that, although this

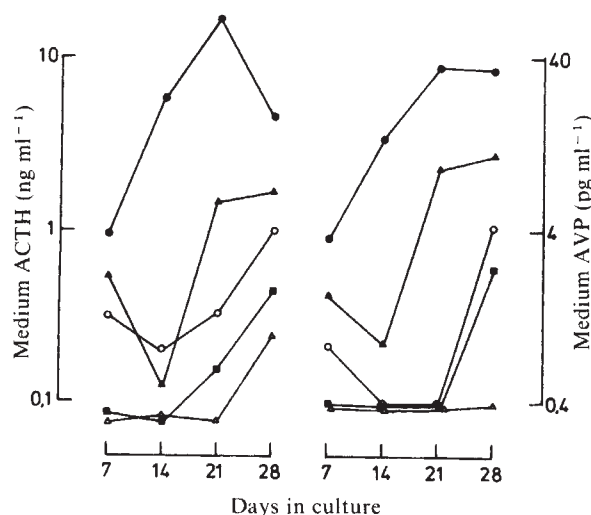


Fig. 3 Bioactive ACTH and immunoreactive AVP content of medium obtained from five individual AP-PV co-cultures as function of time *in vitro*. Tissues are the same as shown in Figs 1 and 2. Each symbol stands for one and the same co-culture. In PV cultures, AVP started to increase in half the cultures during the fourth week *in vitro*, whereas in AP cultures, ACTH gradually decreased from initial levels similar to those observed in AP + PV co-cultures.

increase in ACTH by AVP was far below that induced by co-cultured PV, very low amounts of AVP can exert significant CRF activity in this culture system. In another set of experiments, we determined whether other vasopressinergic cells display similar CRF activity. As illustrated in Fig. 4b, a co-cultured SO explant did not influence ACTH content and secretion by AP (Fig. 4b), whereas the presence of PV again elicited effects on AP qualitatively similar to those shown in the previous culture series.

This study clearly indicates that the PV region, and most probably not the hypophysiotrophic area, is a site for CRF synthesis. Stimulation¹⁷, lesion^{18,19} and histochemical^{20–22} studies have previously implicated the PV in adrenocortical control. It remains to be seen if the CRF released from PV is identical with the one recently described²³, and if AVP is a cofactor in the control of ACTH secretion²⁴ by PV. However, the increased AVP levels in PV-AP co-cultures reveal a remarkable interaction between anterior pituitary and a specific region of the brain, and may have been caused by a known^{25–27} or unknown growth factor from the pituitary or hypothalamus.

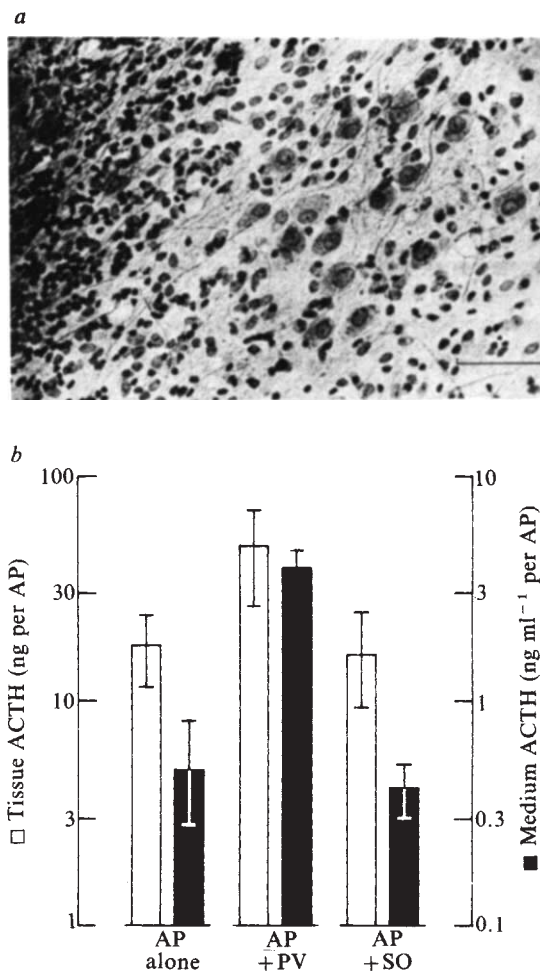


Fig. 4. a, AP-SO co-culture stained by Bodian-silver impregnation after 35 days *in vitro*. Note large cells with fine processes on right, and small anterior pituitary cells on left. Morphological aspects are similar in AP-PV co-cultures, although no morphometric analysis has yet been carried out. Scale bar, 60 μ m. b, Bioactive ACTH content (mean $\pm$ s.e.m.) of tissue (open bars) and medium (solid bars) from five cultures (SO, supraoptic nucleus) after 4 weeks *in vitro*. AVP levels in SO cultures were about half those in PV cultures and, therefore, close to the detection limit. The existence of vasopressin was, however, clearly established by pooling three cultures of SO. The presence of AP had no detectable effect on AVP levels in co-cultures of AP + SO. Protein contents ranged from 180 μ g (three half-anterior pituitaries) to 260 μ g (hypothalamic cultures and co-cultures), and they are not significantly different in the various groups. Results from the set of experiments illustrated in this figure are presented elsewhere²⁸.

Our tissue culture approach opens new ways for identifying additional sites involved in the synthesis of CRF and other hypothalamic factors, and for exploring anterior pituitary actions on the brain.

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Vasoactive intestinal polypeptide enhances muscarinic ligand binding in cat submandibular salivary gland

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The salivary secretion from the cat submandibular salivary gland induced by parasympathetic nerve stimulation is considered to be due to release of acetylcholine (ACh) which binds to muscarinic receptors and activates secretory elements¹. Recent evidence suggests that cholinergic neurones in the cat submandibular gland contain and release another neurotransmitter candidate, vasoactive intestinal polypeptide (VIP)^{2,3}. Although VIP *per se* does not induce salivary secretion, it potentiates the secretion induced by ACh infusions^{2,3}. The present study offers a possible explanation for this potentiation by showing that VIP in physiologically relevant concentrations enhances the binding of muscarinic cholinergic ligands in the cat submandibular gland.

Membranes were prepared from the submandibular salivary gland of adult cats using previously described methods⁴.

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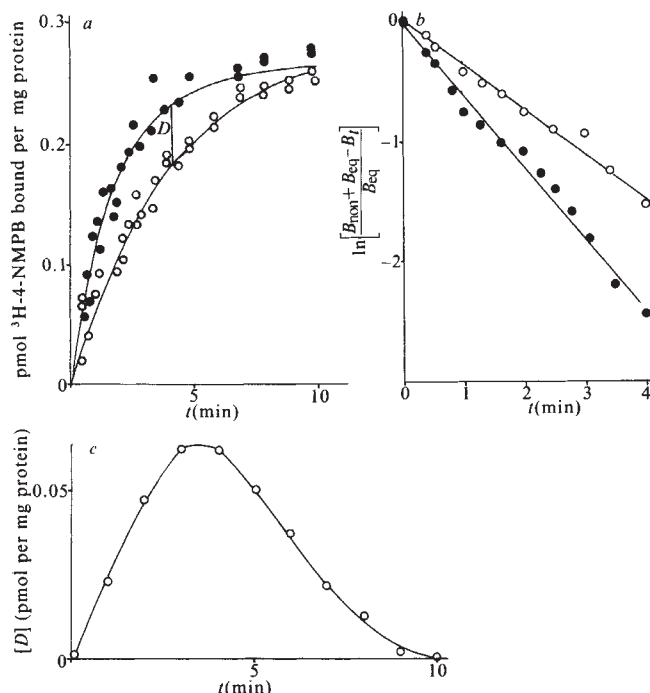


Fig. 1 *a*, Association rate of ^3H -4-NMPB (5.6 nM) to membranes from the cat salivary gland in the absence ($\circ$) and presence ($\bullet$) of VIP (5 nM). Using a loose-fitting glass-Teflon homogenizer at 695 r.p.m., a 10% homogenate (w/v) of the glands in 0.32 M sucrose was prepared by 15 up-and-down strokes. After centrifugation at 1,000g for 5 min the resulting supernatant was subjected to further centrifugation at 10,000g for 40 min. The pellet thus obtained was resuspended in a modified Krebs-Ringer's buffer (137 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl_2 , 1.05 mM MgCl_2 , 5 mM HEPES, 10 μM phenylmethylsulphonyl fluoride, 1 g l⁻¹ glucose) and used in the binding studies without further treatment. All incubations with muscarinic ligands and VIP were carried out at 37 °C and the incubations were started by addition of VIP and ^3H -4-NMPB. Reaction was stopped by filtration through Whatman GF/B glass fibre filters and rinsing of the filters with 10 ml of ice-cold buffer. The filters were counted in a scintillation spectrometer on the next day in toluene/5% diphenyloxazole/0.1% *p*-phenylene phenyloxazole at an efficiency of 45–55%. The experiment was carried out three times with similar results. Data from one representative experiment are shown. Protein was determined according to Lowry *et al.*¹⁷ using bovine serum albumin as standard. The points are the mean ($\pm$ s.e.m.) of triplicate determinations. *b*, The semilogarithmic plot of the association rate data of *a*. These data were analysed by nonlinear least-square fit of the data to the following equation:

$$B_t = B_{\text{non}} + B_{\text{eq}}(1.0 - e^{-kt})$$

where B_t is the total amount ^3H -4-NMPB bound, B_{non} is the nonspecific binding which is equilibrated within seconds, B_{eq} is the amount of ^3H -4-NMPB bound in equilibrium and k is the observed pseudo-first order rate constant. This rate constant is influenced by several factors besides the concentration of ^3H -4-NMPB. The effect of VIP is demonstrated here but not analysed in detail because the VIP effect is transient (*c*), probably due to degradation and because the binding rate of VIP to its receptor is not known, only the onset of its effect on muscarinic binding. *c*, The transient effect of VIP (5 nM) on the association rate of ^3H -4-NMPB (5.6 nM). The difference (*D*) between the curves (*a*) in the presence and absence of VIP is plotted as a function of time.

Muscarinic receptor binding studies were performed using ^3H -N-methyl-4-piperidyl benzilate (^3H -4-NMPB) as the labelled ligand⁵. All incubations were carried out at 37 °C immediately after preparation of the membranes. Highly purified porcine VIP was used in all incubations. The biological activity of the VIP solutions were also tested *in vivo* on cats, by measuring potentiation of the ACh-induced salivary secretion³.

Figure 1*a* and *b* show that the association rate of the muscarinic antagonist ^3H -4-NMPB⁵ at concentrations of 2–15 nM was increased in the presence of VIP (5 nM). This effect was smaller with 50 nM VIP. The effect of VIP was time dependent, being maximal at 3–4 min, and undetectable after 10 min (see Fig. 1*c*). The observed pseudo-first order rate constants⁶ determined for the first 3 min (compare Fig. 1*a* and *b*) were 0.32 ± 0.07 and $0.52 \pm 0.11 \text{ min}^{-1}$, respectively, in the absence and presence of VIP (5 nM). The difference was statis-

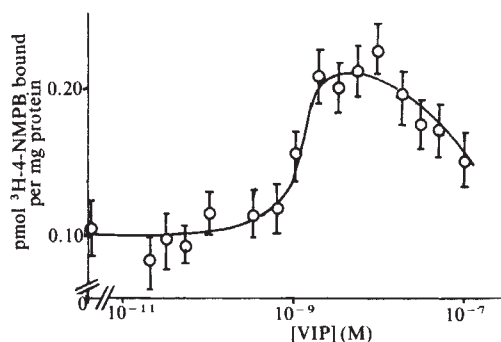


Fig. 2 The effect of VIP on binding to the muscarinic cholinergic receptors. Membranes were incubated with ^3H -4-NMPB (14 nM) and different concentrations of VIP (10^{-11} – 10^{-7} M) for 2 min at 37°C . Data shown are the mean ($\pm$ s.e.m.) of quadruplicate determinations.

tically significant ($P < 0.001$). The dose dependence of the effect of VIP on ^3H -4-NMPB binding was then studied in pseudo-first order conditions. Figure 2 shows that at 2 min of incubation a bell-shaped curve of bound ^3H -4-NMPB versus VIP concentration was obtained, with a maximum between 3×10^{-9} and 3×10^{-8} M VIP. This curve was shifted to the right at longer incubation times, but the effective VIP concentrations were still in the nanomolar range.

At equilibrium conditions (60 min incubation), 0.30 pmol ^3H -4-NMPB per mg protein could be specifically bound both in the absence and presence of VIP, thus suggesting that only the affinity ($K_D = k_{\text{diss}}/k_{\text{ass}}$) and not the number of receptors is changed. The dissociation rate (k_{diss}) for ^3H -4-NMPB from the receptor was determined by starting the measurement after 2 min of incubation with ^3H -4-NMPB by addition of carbamylcholine (10 mM). No effect of VIP (1–50 nM) was detected on the dissociation rate of the ^3H -4-NMPB–receptor complex. Isoprenaline (10^{-7} M), adrenaline (10^{-5} M) and substance P (10^{-7} M), known to influence the function of the submandibular gland, were also tested, but none affected the association rate of muscarinic ligands with the receptor. To determine the tissue specificity of the effect of VIP on muscarinic ligand binding, rat cardiac membranes were tested in similar experiments (the auricles, which are known to have some VIP innervation, were removed). No effect of VIP on the association rate of ^3H -4-NMPB was detected in this preparation.

A 10-nM concentration was chosen from the plateau of the dose-response curve (Fig. 2) to examine the effects of VIP on muscarinic agonist binding. Figure 3a shows that in the presence of VIP, carbamylcholine behaved as a 1,000-fold more potent competitor of ^3H -4-NMPB binding than in the absence of VIP. Note that the slope of binding curves obtained in non-equilibrium conditions (Fig. 3a) is much higher than that in equilibrium conditions (Fig. 3b). The higher slopes may indicate cooperative interactions between carbamylcholine binding sites.

In equilibrium conditions (60 min incubation, Fig. 3b) VIP had no effect on the displacement of ^3H -4-NMPB by carbamylcholine. This 'absence' of effect is probably due to degradation of VIP during the long incubation. Figure 1c shows that the effects of VIP after 10 min have already disappeared, either due to degradation or desensitization. Analysis of VIP after exposure to tissue extracts or plasma indicates that the peptide is rapidly degraded ($t_{1/2} = 1$ – 1.5 min)^{7,8}, suggesting that degradation may be the major factor in the disappearance of its effects. Until stable VIP analogues are available it is not possible to evaluate the contribution of desensitization to disappearance of the effect. A similar disappearance of the effect of VIP over 10–20 min is observed when displacement of ^3H -4-NMPB by another agonist, ACh (10^{-9} – 10^{-2} M), is studied. In the presence of VIP at 5 min incubation time but not with 10 and 20 min incubation, ACh behaves as a 10^5 times more potent agonist ($\text{IC}_{50} \approx 3 \times 10^{-9}$ M, Fig. 3c) than at equilibrium conditions.

The present findings suggest that VIP increases the affinity of muscarinic ACh receptors in the cat submandibular gland.

Under physiological conditions VIP and ACh are probably released simultaneously upon activation of the secretory nerves³. Although VIP by itself does not induce salivation², the findings offer a possible explanation of the potentiating effect of VIP on ACh-induced saliva secretion^{2,3}. Because, in the presence of VIP, the affinity of the muscarinic receptors for ACh increases 10^5 -fold (Fig. 3c) a large increase can be expected in the secretory response which is governed by occupancy of muscarinic receptors¹. Although it is early to speculate about the molecular mechanism of the VIP-mediated increase in muscarinic receptor affinity and association rate, it is relevant that equilibrium binding studies with muscarinic agonists suggest the existence of two or three interconvertible receptor populations having high and low affinity, respectively⁹. It is conceivable that occupancy of VIP receptors induces a shift from low- to high-affinity receptor conformation. (However, note that the data showing an effect of VIP on agonist binding (Fig. 3a, c) were not obtained in equilibrium conditions.)

Some of the muscarinic responses, such as increased cyclic GMP synthesis, seem to be linked to occupancy of low-affinity agonist binding sites, whereas other responses, such as phosphatidylinositol turnover, are apparently linked to both high- and low-affinity binding sites. (See refs 10, 11 for reviews.) Muscarinic responses involved in stimulation of secretion of the lacrimal cells¹², pancreatic cells¹³ and parotid gland¹⁴ relate high-affinity ($K_d < 1 \mu\text{M}$) agonist binding to increased Na^+ permeability. Accordingly, in the salivary gland VIP may switch muscarinic receptors to the high-affinity conformer and thereby increase their efficiency in changing Na^+ permeability and secretion.

The present data suggest that VIP receptors and muscarinic receptors interact in the plane of the membrane. Interaction between different receptors in the same membrane has been reported for muscarinic and β -adrenergic receptors in cardiac membranes¹⁵ as well as for opiate receptors and nicotinic receptors¹⁶ in the adrenal medulla.

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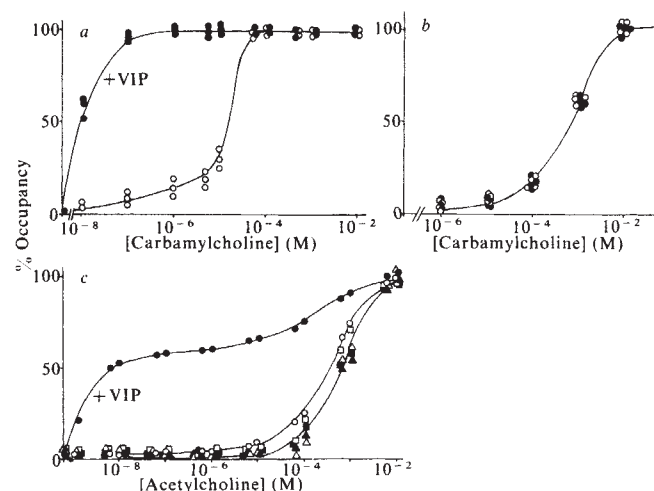


Fig. 3 a, The effect of various concentrations of carbamylcholine on binding of ^3H -4-NMPB (7 nM) in the absence (○) and presence (●) of VIP (10 nM). The samples were incubated for 2 min at 37°C . b, The effect of various concentrations of carbamylcholine on binding of ^3H -4-NMPB (7 nM) in the absence (○) and presence (●) of VIP (10 nM). The samples were incubated for 90 min at 37°C . c, The effect of VIP (10 nM, filled symbols) on displacement of ^3H -4-NMPB (7 nM) by various concentrations of acetylcholine (in the presence of physostigmine, $10 \mu\text{M}$). The displacement curves were run with incubation times of 5, 10 and 20 min. The symbols are: ○, no VIP; ●, +VIP (10 nM); 5 min incubation. △, no VIP; ▲, +VIP (10 nM); 10 min incubation. □, no VIP; ■, +VIP (10 nM); 20 min incubation.

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Neuronal spread of scrapie agent and targeting of lesions within the retino-tectal pathway

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Scrapie is a transmissible encephalopathy of sheep and goats, and a number of mammalian species can be infected experimentally. Various strains of the transmissible agent are distinguishable on pathological and biological grounds in inbred mice^{1–3}. The agents replicate in a variety of tissues, such as those of the lympho-reticular systems^{1,4,5}, but their pathological effects are confined to the central nervous system (CNS). The type, distribution and local intensity of the brain lesions vary, depending mainly on the agent strain, host genotype, route of infection and dose of agent. The principal type of lesion is vacuolar degeneration, typically with a bilaterally symmetrical distribution⁶. (Exceptional reports of its asymmetry can be attributed to 'class III' strains of agent⁷, none of which is used in the present study.) Whether spread of infection around the body involves the migration of infected cells, the agent passing from infected to adjacent uninfected cells, or via a possible viraemic phase to cells remote from infected foci^{8,9}, or transport over considerable distances along nerves, possibly with trans-synaptic infection, is not known. There is evidence that infection spreads in the CNS from the thoracic spinal cord posteriorly and to the brain^{10,11}, but it is still unknown whether the spread is partly or exclusively via neurones, and which types of cell are infected. The finding that the retina became infected following intracerebral injection¹² has been interpreted as evidence for neural spread but this is not conclusive because it can be shown that tracers, for example, can travel from the subarachnoid space into the perineural space of the optic nerve to the epichoroidal and episcleral tissues of the eyeball^{13–15}. I have now investigated whether lesions and infectivity can be targeted into a sensory nucleus following injection into a sense organ on one side of the body with the ipsilateral organ and nucleus acting as controls. The results show that scrapie agent can spread over a considerable distance along a nerve pathway.

The possibility that neural spread of scrapie infectivity could produce targeted lesions was tested by injecting the ME7 strain of scrapie into the right eye of inbred C3H/LacDk mice. Groups of mice were killed for histological examination at intervals of 155–260 days after injection; by 260 days the mice were within 2–3 weeks of the terminal stage of the disease. Haematoxylin-

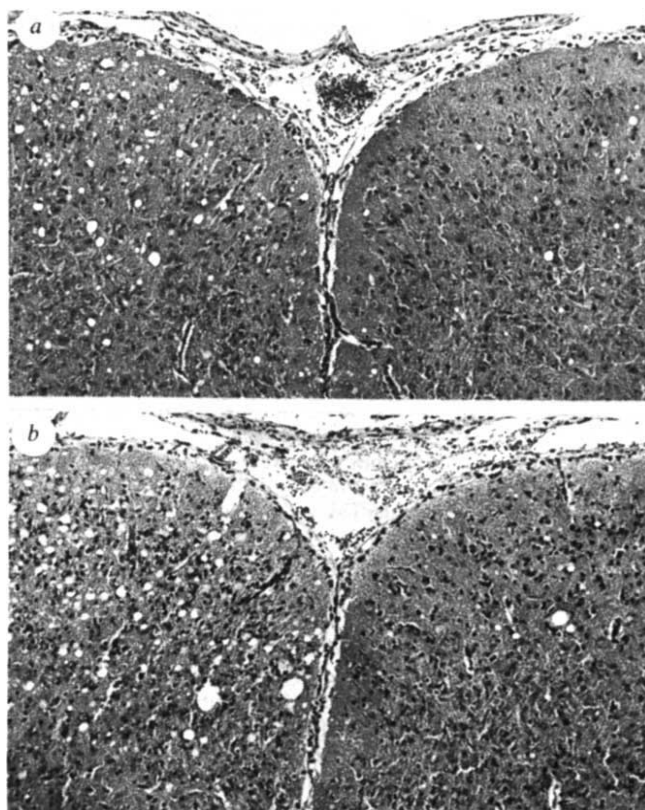


Fig. 1 Asymmetry of vacuolation in the left superior colliculus in C3H mice killed 185 (a) and 190 (b) days after right intraocular injection of ME7 scrapie agent. Haematoxylin and eosin stains used. $\times 78$.

and eosin-stained transverse paraffin sections of their formalin-fixed brains were examined, using a standard procedure¹⁶. In mice killed at the earlier intervals, asymmetrical lesions were confined to the contralateral (left) superior colliculi and no lesions were apparent elsewhere in the brain. This tectal asymmetry continued throughout the series (Fig. 1a), becoming less frequent as lesions developed symmetrically in both the tectum and other regions of the brain. By 250 days post-injection only symmetrical lesions were present (Table 1). From about 70% of the way through the incubation period of 265 ± 3 (mean $\pm$ s.e.) days, an increasing proportion of cases showed a form of tectal asymmetry having severe degeneration of the contralateral side, but much less involvement of the ipsilateral superior colliculus (Fig. 1b), and low-grade symmetrical lesions elsewhere in the brain.

Retinal ganglion cells are known to project to and synapse with various brain nuclei, including the superior colliculi and lateral geniculate bodies¹⁷. In a few cases, low-grade vacuolation of the contralateral lateral geniculate nucleus occurred, but never with the consistency or severity seen in the optic tectum. This prominent asymmetry in the superior colliculi but not in the lateral geniculate nuclei is readily explained on several grounds. First, when injection routes which give fully symmetrical lesions (such as intracerebral or intraperitoneal injections) are used the ME7 agent in C3H mice produces severe vacuolation in the superior colliculi but only mild vacuolation in the lateral geniculate nuclei. Second the lateral geniculate is only a small homogeneous structure in rodents compared with its greater importance and complexity in carnivores and primates¹⁷; and third, there is more decussation to the superior colliculi than to the lateral geniculate nuclei¹⁸—90% to the latter in the mouse¹⁹.

The observed distribution of infectivity in dissected optic nerves and superior colliculi is entirely in accord with the lesion data. Following injection into the right eye, at 68 days infectivity

Table 1 Numbers of C3H mice with vacuolar degeneration in the superior colliculi, at intervals after intraocular injection into the right eye with ME7 scrapie agent

	Days post-injection									
	155	161	167	185	190	200	216	232	251	
Asymmetry of vacuolation in superior colliculus										
Left	2	4	2	2	1	2	0	1	0	
Right	0	0	1*	0	0	0	0	0	0	
Symmetrical lesions	1	0	1	0	0	1	2	0	2	
No lesions	1	1	0	0	0	0	0	0	0	
Incidence of asymmetry	2/4	4/5	3/4	2/2	1/1	2/3	0/2	1/1	0/2	

Inoculum of scrapie agent was 10^4 – 10^5 ICLD₅₀ units per 0.002 ml of a 500g supernatant of terminal ME7-affected C57BL mouse brain homogenate.

* Data are available for 78 mice with asymmetrical vacuolation in the optic tectum following intraocular injection with scrapie from these and other unpublished experiments. This is the only deviant result in terms of sidedness and the possibility that the block was inverted during the preparation of sections cannot be excluded.

is present in its optic nerve and tract, and in the contralateral superior colliculus to which it projects, but absent from the optic nerve and tract from the left eye and from the ipsilateral superior colliculus. At 105 days there is 500 times more infectivity in the contralateral than the ipsilateral superior colliculus. We now have preliminary results from a much wider time series which confirms that these findings are part of a consistent trend and that the infectivity appears in the contralateral superior colliculus several weeks before the lesions.

The results reported here provide clear evidence that ortho-grade axonal spread can occur in scrapie in the CNS, which could account for the differences in titre found for the peripheral and central nervous systems, all pointing to neural spread^{10–12}. This is also consistent with the recent evidence that the sympathetic system is a mode of entry of peripherally replicated scrapie into the thoracic spinal cord²⁰. The extensive cross-over of retinal ganglion cell axons which occurs in adult rodents^{19,21} would, if neural spread occurred, result in the targeting of the earliest lesions largely in the contralateral superior colliculus, as has been demonstrated with tracers of axoplasmic flow such as ³⁵S-methionine and horseradish peroxidase^{22–24}. The alternative possibilities, of spread of infection via cell-to-cell contact in epineural glial cells, or of spread by extension through the subarachnoid space around the optic nerves and tracts, are clearly inconsistent with these results as neither method of spread would produce contralateral asymmetry restricted to the optic tectum. As an analogue of a central nervous pathway²⁵, the evidence for spread in the retino-tectal projection suggests that spread in the CNS may involve a wider range of central axons than previously thought, but does not rule out the possibility of other modes of spread, such as via the extracellular space and within and between glia.

It is now necessary to extend the observations described here, which are limited to the ME7 strain of scrapie agent, to other agents with different pathological manifestations, such as 79A agent in which white matter vacuolation is prominent²⁶, compared with 22C, where grey matter is involved exclusively, to test whether the targeting within the optic pathway can be shown to be even more specific.

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Immune interferon release when a cloned cytotoxic T-cell line meets its correct influenza-infected target cell

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T cells stimulated by mitogens¹, specific antigens², including viral antigens³, and alloantigens⁴ have been shown to produce immune interferon (IFN- γ). However, the exact circumstances of induction of IFN- γ in T cells have not yet been established, particularly the precise nature of the cell type producing the interferon. We have recently described⁵ the selection and cloning of a BALB/c cytotoxic T(T_c)-cell line (L4) which grows in the presence of T-cell growth factor (TCGF), is Lyt-2 positive and kills H-2^d target cells infected with any type A influenza virus. The aim of the present study was to examine whether this cloned cytotoxic T-cell line produced IFN- γ on contact with the appropriate target cell. It was found that small amounts of IFN- γ were produced on co-culture of L4 with target cells, whereas L4 cells on their own released no detectable interferon. The pattern of antigenic specificity by L4 cells for interferon production resembled the requirements for killing in that T_c cell mediated lysis was restricted to H-2^d targets infected with influenza A, but not influenza virus B strains, and interferon production was not prevented by a monoclonal antibody to the virus' haemagglutinin, which does not inhibit cytotoxicity while neutralizing the virus.

L4 T_c cells were maintained in RPM1 1640 medium containing 10% fetal calf serum, ampicillin, kanamycin and 10% RS (supernatant from rat spleen cells activated by concanavalin A)⁵. The cytotoxicity of L4 declined rapidly during *in vitro* passage and regular recloning was necessary to maintain the line. ⁵¹Cr-release cytotoxicity assays were carried out at regular intervals to survey cytotoxicity.

To examine interferon production, L4 T_c cells were incubated with A/X31-infected P815 (H-2^d) target cells in the same conditions as the cytotoxicity assay except that the target cells were unlabelled. After 6 h incubation the cells were centrifuged lightly and interferon levels in the supernatant medium determined by titration on C3H10T $\frac{1}{2}$ mouse fibroblasts⁶ using the inhibition of nucleic acid synthesis method⁷ with Semliki Forest virus as challenge virus. Table 1 shows that L4 T_c cells produce

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interferon on contact with A/X31-infected P815 cells, but that L4 T_c or infected P815 cells incubated alone for the same time period (6 h) do not. A monoclonal antibody (3/1) to the H3 haemagglutinin of A/Memphis/71, which neutralizes A/X31 virus infectivity⁸ but does not block killing⁹, does not prevent interferon production. P815 cells coated with UV-irradiated A/X31 virus, which do not act as targets, do not induce interferon. A/X31 virus alone added to L4 T_c cells at the same concentration used to infect P815 cells does not induce interferon in L4 T_c cells. Thus, production of interferon by L4 T_c cells required interaction with infected targets. The presence of free infectious virus was neither necessary nor sufficient for interferon production.

We next determined whether the production of interferon was related to the recognition of appropriate antigens on the target cells. P815 cells infected with either A/X31 or A/USSR both act as targets for L4 T_c cells⁵ and both induce interferon production (Table 2). P815 cells infected with B/HK, on the other hand, neither serve as targets for L4 T_c cells⁵ nor induce interferon (Table 3).

Table 1 Cytotoxic T-cell clone L4 releases interferon on contact with targets infected with influenza strain A/X31

L4 T _c cells	P815 targets virus infection	Antiserum to HA*	Interferon production (U ml ⁻¹)	% Target cell lysis at K/T ratio	
				5/1	2.5/1
+	A/X31	—	100	29.4	27
+	—	—	<3	—	—
—	A/X31	—	<3	—	—
+	A/31	1/100	120	29	26.5
+	UV A/X31	—	<3	—	—

The cytotoxicity assay was carried out as previously described¹⁷. In brief, P815 cells infected for 1.5 h with 800 haemagglutinin units of virus and labelled with ⁵¹Cr were washed three times and plated out at 10⁴ per well in 96 round bottom microtitre plates. After 6 h incubation with L4 T_c cells in 0.2 ml at 37 °C, the plates were centrifuged for 5 min at 1,000 r.p.m.; 0.1-ml samples were collected and radioactivity estimated in a γ -counter. %⁵¹Cr release = c.p.m. (experimental) — c.p.m. (spontaneous release)/c.p.m. (total) — c.p.m. (spontaneous release). K/T, killer/target cell ratio. To test the production of interferon, the assay was carried out in the same way in 0.2 ml per well except that the infected target cells were not labelled with ⁵¹Cr, and K/T was 10:1 or 5 × 10⁵ T_c cells per ml. The supernatant medium was collected and interferon assayed on C3H10T_{1/2} cells⁷ by the INAS₅₀ method⁷. Briefly, replicate monolayers of cells were treated overnight with dilutions of interferon. The cells were then challenged with Semliki Forest virus and the extent of virus replication directly assessed by labelling with ³H-uridine in the presence of actinomycin D to suppress cell RNA synthesis. Titres are quoted in units per ml and are approximately equivalent to international units of mouse IFN- α + β (there is at present no standard for mouse IFN- γ).

* Monoclonal antibody (3/1) to haemagglutinin of A/X31⁸ which does not inhibit T-cell killing, but neutralizes infective virus⁹.

We further correlated the production of interferon by L4 T_c cells with their ability to recognize and kill infected H-2^d targets but not infected H-2^b cells or infected H-2^{dm2} cells (B.A.A. and Y-L.L. in preparation). The dm₂ mutant lacks H-2L and H-2M molecules in the D region of the major histocompatibility locus. To test the killing of virus-infected cells derived from different mouse strains it was necessary to use lipopolysaccharide-stimulated B lymphoblasts as targets, which are not so efficiently killed by L4 T_c cells as P815 tumour cells. IFN- γ production was therefore low, but the amounts produced paralleled the killing obtained in cytotoxicity assays. Thus, whereas A/X31-infected BALB/c (H-2^d) lymphoblasts were killed by L4 T_c cells and induced interferon production, A/X31-infected lymphoblasts from C57/B16 (H-2^b) or BALB/c dm₂ mice (H-2^{dm2}) were neither killed by L4 T_c cells nor induced interferon (data not shown).

The type of interferon produced by L4 T_c cells interacting with A/X31-infected P815 cells was determined by antiserum neutralization and acid lability tests. Two antisera were used: a sheep antiserum to interferon produced by induction of C243 cells, and a rabbit antiserum to interferon produced by induction of mouse L cells (obtained from the NIH). Both antisera neu-

Table 2 Cytotoxic T-cell clone releases interferon on contact with targets infected with heterologous type A influenza virus

L4 T _c cells	P815 targets virus infection	Interferon production (U ml ⁻¹)
+	A/X31	45
+	A/USSR	50
—	A/X31	<3
—	A/USSR	<3

Experimental conditions were as described in Table 1 legend. The K/T ratio for interferon production was 8:1. At a K/T of 5:1, L4 cells lysed 17% of A/X31-infected and A/USSR-infected target cells.

tralize both mouse IFN- α and IFN- β but not IFN- γ . We found that both antisera failed to neutralize significantly the interferon produced by L4 T_c cells in conditions where interferon produced by Newcastle disease virus-induced mouse L cells (a mixture of IFN- α and IFN- β)¹⁰ was completely neutralized. IFN- γ is unstable at pH 2 whereas IFN- α and IFN- β are both stable¹¹, the interferon produced by L4 T_c cells was completely destroyed by pH 2 treatment. Therefore by both these criteria the interferon produced was IFN- γ .

Recently Marcucci *et al.*¹² have tested several T-cell clones, growing in TCGF, for IFN- γ production after mitogen stimulation. These clones, derived from AKR mice sensitized with picryl chloride, produced up to 800 units of IFN- γ per 10⁶ cells. All the clones were Thy-1.1 antigen positive; but since they lacked detectable Lyt markers it was not possible to determine to which T-cell subpopulation they belonged. Our data indicate that Lyt-2-positive, target-specific T_c cells produce IFN- γ on contact with their target cell. There is a previous report of production of lymphokines by T cells of both Lyt-1- and Lyt-2,3-positive phenotype¹³. The characteristics of IFN- γ production resemble closely the characteristics of killing in viral antigenic specificity and H-2 restriction. As the virus alone does not induce IFN- γ , it seems that for IFN- γ production the L4 T_c cells need to 'see' the viral antigen in the context of cellular antigens. Accessory cells (such as macrophages) seem unnecessary for IFN- γ induction, although it is possible that they may augment interferon production and our data do not exclude the possibility that other T-cell subpopulations also produce interferon on appropriate stimulation. We also cannot exclude the possibility that the interferon is produced by the target cells following specific recognition by the T_c cell, but we think this unlikely because the interferon is type γ .

The ability of T_c cells to produce interferon when they meet their histocompatible virus-infected target cell is of considerable importance. Immune T cells have been shown to protect against influenza virus infection¹⁴, and clone L4 cells have been shown to protect against lethal influenza infection¹⁵ and to reduce the replication of influenza virus in the lungs of infected mice. It was thought that T_c cells protect by killing cells early after virus

Table 3 Influenza type B virus-infected target cells do not induce interferon release by L4 cytotoxic T cells

L4T _c cells	Virus infection of P815 targets	Interferon (U ml ⁻¹)	Target cell lysis at K/T ratio	
			4:1	2:1
+	A/X31	120	20.3	14.5
—	A/X31	<3	—	—
+	B/HK	<3	2.1	0.8
+	—	<3	—	—
—	B/HK	<3	—	—

Assay conditions as in Table 1. L4 T_c cells ± influenza-infected P815 targets were incubated in a microtitre plate for 6 h at K/T ratio of 9:1 (10⁵ target cells per well). Cells were centrifuged and the supernatant analysed for interferon. The data show interferon released by 4.5 × 10⁵ L4 cells per ml over 6 h. A parallel assay was carried out with ⁵¹Cr-labelled and A virus-infected target cells to measure the % ⁵¹Cr released during a 6 h incubation at K/T ratios as shown.

infection, before virion assembly but after the expression of viral proteins on the cell surface. Our finding that L4 T_c cells produce IFN- γ after contact with their target adds another dimension to the role of T_c cells in preventing spread of virus. The IFN- γ produced would reduce spread of virus released from infected cells before killing occurred, and could also activate other immune effector cells, such as natural killer cells¹⁶, which may be involved in resistance to virus infections. It could also serve to protect the T_c cells themselves from infection.

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Phorbol-12,13-diester 12-ester hydrolase may prevent tumour promotion by phorbol diesters in skin

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Tumour promoters are not themselves carcinogenic but can induce tumours in mice treated previously with an otherwise insufficient dose of certain chemical carcinogens^{1–6}. One of the most potent tumour-promoting agents is 12-O-tetradecanoyl-phorbol-13-acetate (TPA), initially isolated from croton oil⁷. The effects of tumour promoters are species specific⁸; croton oil, of which TPA is the most active component, does not act as a tumour promoter in rat, rabbit, guinea pig or hamster skin in conditions for which it is a potent tumour promoter in mouse skin^{8–12}. Recently, we have purified the enzyme phorbol-12,13-diester 12-ester hydrolase (PDEH) to homogeneity from mouse and human liver cytosol¹³. This enzyme converts biologically active phorbol-12, 13-diester to the inactive phorbol-13-monoester¹³ in a dose-, time- and temperature-dependent manner. PDEH has been partially purified from hamster skin but is not detected in mouse skin, suggesting that the basis for the TPA tumour promotion only in mouse skin is a result of differences between the species in levels of TPA inactivating enzyme (PDEH) in the skin. We demonstrate here that responsiveness to TPA-induced tumour promotion is related to the level of PDEH activity in skin of all animals tested.

TPA elicits various biological and biochemical responses *in vitro* as well as *in vivo*^{1–6,14–18}. High-affinity specific receptors for biologically active phorbol and ingenol esters have been reported for a wide variety of cells in culture as well as in animal tissues^{19,20}. The lack of responsiveness of certain animal species

Table 1 Distribution of PDBu binding sites in skin of various species

Species	³ H-PDBu binding (ng per mg protein)
Mouse (BALB/c)	0.38
Rat (Fisher)	0.52
Guinea pig (N-2)	0.35
Hamster (Syrian)	0.23
Rabbit (NZW)	0.35
Cat	0.41
Dog	0.42
Rhesus monkey	0.37
Human	0.32

The shaved skins from adult animals were minced and suspended in 5 vol of cold phosphate-buffered saline (PBS) and disrupted with a polytron PT-10 (Brinkman) at 21,000 r.p.m. at 4 °C for 180 s. Four 30-s pulses at 30-s intervals were used. The crude homogenate was centrifuged at 105,000g for 1 h at 4 °C. Human skin from an autopsy specimen of a 5-yr-old female accident victim was similarly processed. The supernatant was removed and used for PDEH assay. The pellet was re-suspended in the original volume of cold PBS. The binding of ³H-PDBu to crude homogenate or pellet suspension was performed in duplicate in 12 × 75 mm disposable glass tubes (Kimax) in the absence or presence of 20 µg ml⁻¹ unlabelled PDBu. The binding mixture contained 5 ng ³H-PDBu (~4 × 10⁴ c.p.m.), 0.2% final concentration of dimethyl sulphoxide (DMSO) and several concentrations of crude homogenate or pellet suspension in a total volume of 0.25 ml binding buffer (BB) consisting of Dulbecco's minimum essential medium (DMEM) containing bovine serum albumin (BSA, 1 mg ml⁻¹) and N,N-bis-(2-hydroxyethyl)-2-amine-ethane sulphonic acid (BES, 5 mM), adjusted to pH 6.8. After incubation at 23 °C for 30 min, tubes were chilled, 0.25 ml cold 4% calf serum (Colorado Serum Corporation) and 0.7 ml cold 25% polyethylene glycol (PEG 6,000) in 1 mM Tris-HCl pH 7.4 were added to each tube, the contents vortexed and the tubes allowed to stand at 4 °C for 15 min before being centrifuged (1,500g for 10 min at 4 °C). The supernatant solution was carefully drained off, the pellet resuspended in 2.5 ml of cold 10% PEG in 0.1 M Tris-HCl pH 7.4, and the tubes were again centrifuged as described above. The supernatant solution was removed and pellets solubilized in 0.8 ml of lysing buffer (0.01 M Tris-HCl pH 7.4, containing 0.5% SDS and 1 mM EDTA). The mixture was transferred to counting vials and 10 ml Aquassure (NEN) added to each vial. The vials were shaken vigorously and radioactivity determined using a Beckman β counter. The radioactivity bound in the presence of 20 µg ml⁻¹ (50–200 c.p.m.) unlabelled PDBu was considered to be nonspecific and all data were corrected accordingly. Protein concentration was determined as described elsewhere²⁷. The animal strains used are given in parentheses.

to two-stage tumorigenesis is apparently not due to the lack of initiating carcinogenic activity because rabbit and hamster are highly susceptible to skin carcinogenesis caused by polycyclic aromatic hydrocarbons^{21,22}.

The phorbol diester inactivating enzyme (PDEH) is a single-chain, hydrophobic glycoprotein with a molecular weight (MW) of 60,000. It has an isoelectric point (pI) of 5, exhibits optimum activity at pH 7.5–8.5, and is heat- and acid-labile. Phenyl-methylsulphonyl fluoride (PMSF) is a potent inhibitor of the enzyme, as are zinc, cobalt and fluoride ions. The amino acid composition and certain properties of the enzyme are different from purified porcine liver carboxy esterase (EC 3.1.1.1)^{23,24}. The natural substrate(s) of PDEH are unknown; however, porcine liver esterase (Sigma) unlike PDEH, does not inactivate phorbol diester.

To investigate the restriction of TPA action in nonresponsive species, we studied the specific receptors for biologically active phorbol esters^{19,20} and PDEH activity in skin of various species. Enzyme activity was measured by incubating ³H-PDBu (phorbol-12,13-dibutyrate) and tissue extract at 37 °C for 30 min and then measuring the amount of remaining ³H-PDBu by its binding to mouse brain membranous fraction (BMF), the richest source of receptors²⁰, for 30 min at 23 °C. We found that skin from all species tested contains TPA receptors, and that PDEH activity is absent from normal mouse skin, but present in very high levels in hamster, rat, guinea pig and rabbit skin. The distribution of PDBu binding sites in skin of various species is given in Table 1. Skin from all species (mouse, rat, guinea pig, hamster, rabbit, cat, dog, monkey and human) bound between 0.32 and 0.52 ng PDBu per mg protein. Thus, responsive and nonresponsive animals have roughly comparable levels of TPA receptors in their skin. Therefore, the lack of responsiveness to TPA characteristic of most rodent species except the mouse, is not due to the absence of specific TPA receptors.

Figure 1 shows that extracts from hamster, rat, guinea pig or

Table 2 Distribution of phorbol diester-12-esterase activity in skin of various species

Skin extract	Activity (³ H-PDBu cleaved; ng per mg protein)	TPA responsiveness (skin carcinogenesis)
Mouse (<i>nu</i> ⁻ / <i>nu</i> ⁻)	<0.1	++
Heterozygote mouse (<i>nu</i> ⁺ / <i>nu</i> ⁻)	<0.1	NK
Nude mouse (<i>nu</i> ⁺ / <i>nu</i> ⁺)	1.7	-
Rat	22.7	-
Guinea pig	13.9	-
Hamster	100.8	-
Rabbit	7.8	-
Cat	<0.1	NK
Dog	<0.1	NK
Rhesus monkey	<0.1	NK
Human	0.1	NK

NK, not known. -, With low doses of TPA¹⁵.

rabbit readily inactivated ³H-PDBu, reducing its binding to the specific receptors in a dose-dependent manner. In contrast, extracts from mouse, cat, dog, monkey and human skin do not affect ³H-PDBu binding to receptors. Failure of nude mouse skin to respond to low doses of TPA has been reported elsewhere²⁵. Interestingly, nude mouse skin extracts contain small but significant amounts of PDEH whereas heterozygote mouse skin extracts, like wild-type mouse skin extracts, do not express any activity (Fig. 1). The levels of PDEH enzyme activity in skin extracts from various species are shown in Table 2. Hamster, rat, guinea pig, rabbit and nude mouse extracts cleaved, respectively, 100.8, 22.7, 13.9, 7.8 and 1.7 ng PDBu per mg protein, in our assay conditions. Mixing experiments demonstrated that the mouse skin extract contains a low level of a PDEH inhibitor, which can slightly decrease the hamster enzyme activity.

Table 3 summarizes a HPLC analysis of ³H-PDBu treated with skin extract from various species. Extracts from hamster, rat, guinea pig and rabbit skin converted ³H-PDBu to ³H-13-PB (phorbol-13-butyrate) while there was no reduction in ³H-PDBu nor appearance of other metabolites in samples treated with mouse, cat, dog, monkey or human skin extracts. HPLC analysis of ³H-PDBu metabolite(s) (24 h after PDBu application) from mouse, hamster and guinea pig skin *in vivo* revealed that all the radioactivity recovered from mouse skin co-elutes with PDBu, whereas ~80% of the labelled material from hamster and ~96% radioactivity from guinea pig skin co-elutes with 13-PB, the rest remaining as PDBu. Thus, the

Table 3 HPLC analysis of ³H-PDBu treated with skin extract from various species

Species	% Radioactivity eluted as	
	PDBu	13-PB
Control	100	0
Mouse	100	0
Rat	0	100
Guinea pig	2.6	97.4
Hamster	0	100
Rabbit	35.2	64.8
Cat	100	0
Dog	100	0
Rhesus monkey	100	0
Human	100	0

³H-PDBu (120 µl; ~16 × 10⁴ c.p.m.) in BB, 0.2% final concentration DMSO and 2 mg of skin extract protein in 0.8 ml PBS were incubated for 1 h at 37 °C. ³H-PDBu and products were extracted with a chloroform-methanol mixture (2:1), the organic phase containing almost all radioactivity dried under nitrogen, the material dissolved in 25 µl DMSO. Ten microlitres from each sample were analysed by HPLC using C₁₈-Bondapak/Porasil (Waters Associates)¹³.

direct application of PDBu to skin gave the same results as those obtained using skin extracts in an *in vitro* assay.

Our results show a close relationship between levels of PDEH activity in skin and responsiveness to TPA-induced tumour promotion in skin in all cases (mouse, hamster, rabbit, guinea pig and rat) for which data are available. Thus, TPA susceptibility of mouse skin, but not of other animal skin, may be due to the lack of expression of PDEH in this tissue. The high levels of PDEH in a tissue would inactivate TPA or other active phorbol esters, leading to insensitivity of that tissue to TPA-induced tumour promotion. On the basis of this observation, one would predict that TPA and related active diterpene esters would act as tumour promoters in cat, dog, monkey and human skin. An effective inhibitor of PDEH such as PMSF¹³ would potentiate the action of TPA in skin and might make TPA-nonresponsive animals become sensitive to TPA. Conversely, inducers of the enzyme might render a responsive animal nonresponsive.

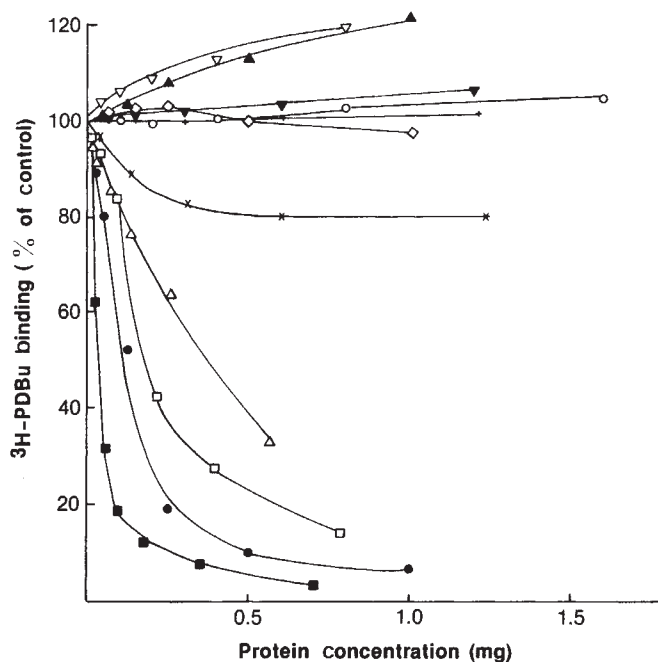


Fig. 1 Effects of protein concentration in skin extracts from various species on ³H-PDBu inactivation. ³H-PDBu (5 ng in 30 µl BB buffer), 0.2% final concentration DMSO and 0–200 µl of skin extract in PBS in a final volume of 0.23 ml were incubated in duplicate at 37 °C for 30 min. Tubes were placed on ice and 20 µl of murine BMF in PBS (50 µg protein) were added to each tube, the contents of the tubes were mixed and incubated at 23 °C for 30 min. After this time PDBu binding was determined as described in Table 1; 100% binding represents ~5,000 c.p.m. ○, Inbred mouse (*nu*⁻/*nu*⁻); +, heterozygote mouse (*nu*⁺/*nu*⁻); ×, nude mouse (*nu*⁺/*nu*⁺); ●, rat; □, guinea pig, ■, hamster; △, rabbit; ▲, cat; ▽, dog; ▼, rhesus monkey; ◇, human.

Increasing the dose of TPA and/or TPA treatment frequency would probably enhance the susceptibility to TPA-elicited tumour promotion in PDEH-positive tissues. In addition, those tumour promoters which do not contain an ester group, such as dihydroteleocidin B²⁶, should be refractory to the action of PDEH and concomitantly, should act as promoting agents in PDEH-positive tissues, provided that they are not inactivated by some other enzyme in the tissue. Although PDEH is not expressed in mouse skin, very high amounts of the enzyme can be isolated from mouse liver and kidney. One would expect these tissues, like hamster, rat, rabbit or guinea pig skin, to be refractory to the various actions of the TPA-related tumour promoters.

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Calmodulin confers calcium sensitivity on secretory exocytosis

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The cortical reaction at fertilization of a sea urchin egg results in the elevation of the fertilization membrane due to the exocytosis of cortical granules lying directly beneath the plasma membrane. These secretory granules are vesicles $\sim 1 \mu\text{m}$ in diameter and can be easily observed in phase-contrast optics. Insemination of the egg results in the release of intracellular ionized calcium which acts as the trigger for the fusion of the cortical granules with the plasma membrane and the expulsion of their contents into the extracellular space^{1,2}. Although Ca^{2+} is known to be involved in exocytosis, it has not yet been clearly demonstrated how the high sensitivity to calcium is conferred on exocytosis^{3,4}. Here we examine the action of antibodies to calmodulin on the secretion of the cortical granules and demonstrate that calmodulin is responsible for the high calcium sensitivity of the cortical reaction and can be localized on the inner plasma membrane surface.

Baker and Whitaker have previously shown that the calcium sensitivity for cortical granule fusion with the plasma membrane is the same for both intact eggs and their *in vitro* preparation of isolated cortical surfaces⁵. Furthermore, they could stabilize the micromolar calcium thresholds for exocytosis by keeping the isolated surfaces in media containing millimolar concentrations of ATP⁵. They also demonstrated a strong inhibition by the antipsychotic drug trifluoroperazine, which suggested that a modulatory calcium-binding protein was involved in exocytosis⁶.

Calmodulin is a ubiquitous calcium-binding protein used in many regulatory pathways and has been found in sea urchin eggs where it is known to activate NAD kinase⁷⁻¹⁰. The *in vitro* preparation for exocytosis allowed us to test directly the effects of antibodies to calmodulin without complications from poisoning other enzyme systems. Commercially obtained antibody to electrophoretically homogeneous native rat testis calmodulin was produced in sheep and the calmodulin-specific IgG was purified from other sheep immunoglobulins and proteins by affinity chromatography on calmodulin-Sepharose¹¹. Isolated cortical surfaces were prepared on poly-lysine-coated glass coverslips in the form of small (5 μl) chambers which could be directly observed with a Zeiss phase microscope while solutions were changed. These chambers were then incubated at 100% humidity and 16 °C in the isolation medium either alone or with

calmodulin antibody or preimmune serum or calmodulin antibody plus excess calmodulin (purified from bovine testis)¹². At the end of the incubation period, each chamber was photographed before and 60 s after testing with a single calcium concentration and the number of reacted cortical granules was counted.

When tested with the isolation medium alone, the calcium sensitivity of exocytosis remained constant for all periods of incubation with $>70\%$ discharge of the cortical granules within 60 s at $5 \mu\text{M Ca}^{2+}$. Short periods of incubation (45 min) with the calmodulin antibody had no inhibitory effect but inhibition rapidly set in with longer periods, with the calcium threshold rising to $150 \mu\text{M Ca}^{2+}$ at 65 min and complete block even to 1 mM Ca^{2+} at 75 min. Figure 1 shows the results obtained with 75-min incubations, demonstrating the complete block induced by treatment with calmodulin antibody. In contrast, controls with isolation medium alone, preimmune serum or calmodulin antibody preincubated with excess calmodulin (at a ratio of 7 mol of calmodulin to 1 mol antibody) retain their high sensitivity to Ca^{2+} . A complete reversal of this block could be obtained by a further 10 min of treatment with calmodulin at a concentration of $2 \times 10^{-5} \text{ M}$ ($n = 3$). The rapid onset of the block after a delay and the much shorter period required for reversal with calmodulin suggests that a large excess of calmodulin is associated with each cortical granule-plasma membrane complex with only a relatively few calmodulin sites necessary for full calcium sensitivity and discharge at $5 \mu\text{M Ca}^{2+}$. If this

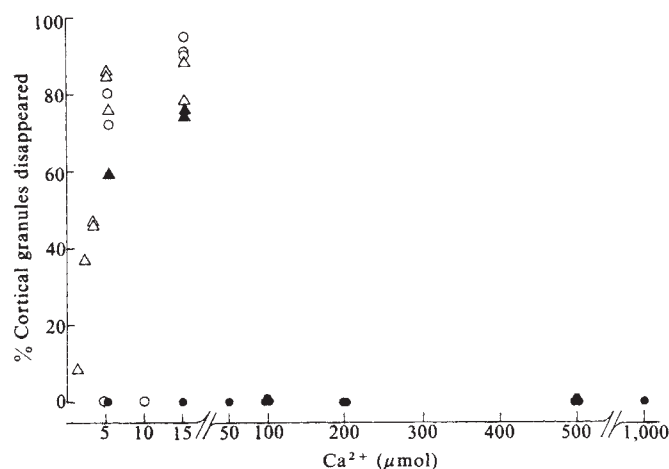


Fig. 1 Influence of antibody to calmodulin on the calcium sensitivity of exocytosis in isolated cortical surfaces. Δ , Controls in which the cortices were incubated in the isolation medium alone. $\blacktriangle$, Cortices incubated in isolation medium containing $750 \mu\text{g ml}^{-1}$ protein preimmune serum. $\circ$, Cortices incubated in isolation medium containing $400 \mu\text{g ml}^{-1}$ sheep antibody to calmodulin plus $2 \times 10^{-5} \text{ M}$ bovine testis calmodulin¹². This represents a molar ratio of 7 calmodulin to 1 sheep IgG. Antibody was preincubated with calmodulin for 1 h before being applied to the cortices. $\bullet$, Cortices incubated in isolation medium containing $400 \mu\text{g ml}^{-1}$ calmodulin antibody. Eggs of the sea urchin *Lytechinus pictus* were obtained as previously described¹. Isolated cortical surfaces were prepared by the method of Baker and Whitaker⁵ by sticking portions of egg cortices on glass coverslips with poly-DL-lysine in the following isolation medium: 250 mM potassium gluconate, 500 mM glycine, 10 mM NaCl, 10 mM HEPES, 10 mM EGTA, 5 mM Mg-ATP, 1 mM free Mg^{2+} , 10^{-7} free Ca^{2+} , pH 6.7. Ionized calcium and magnesium concentrations were calculated from the stability constants for EGTA¹⁴. The abscissa represents the ionized calcium concentration in the test solutions which were otherwise identical to the isolation medium except that no attempt was made to buffer calcium with EGTA at Ca^{2+} concentrations above $15 \mu\text{M}$. Solution changes were made by placing a fragment of filter paper at one open end of the small (5 μl) glass coverslip chamber containing the cortical surfaces and flushing gently 20 μl of new solution through from the other end; this was repeated three times. After a 75-min incubation period at 16 °C, each chamber was viewed with a Zeiss inverted microscope (IM35) using a 63 power oil immersion phase objective. Each cortical preparation was photographed before and 60 s after the introduction of the test solution, with the time being measured from the time of the second flush of new solution. Preimmune serum and antibody to calmodulin were obtained from CAABCO, Houston, Texas. This non-precipitating sheep calmodulin antibody demonstrates cross-reactivity to calmodulin from vertebrates, invertebrates and plants, and exhibits $<0.2\%$ cross-reactivity to troponin C and no cross-reactivity to parvalbumin¹¹.

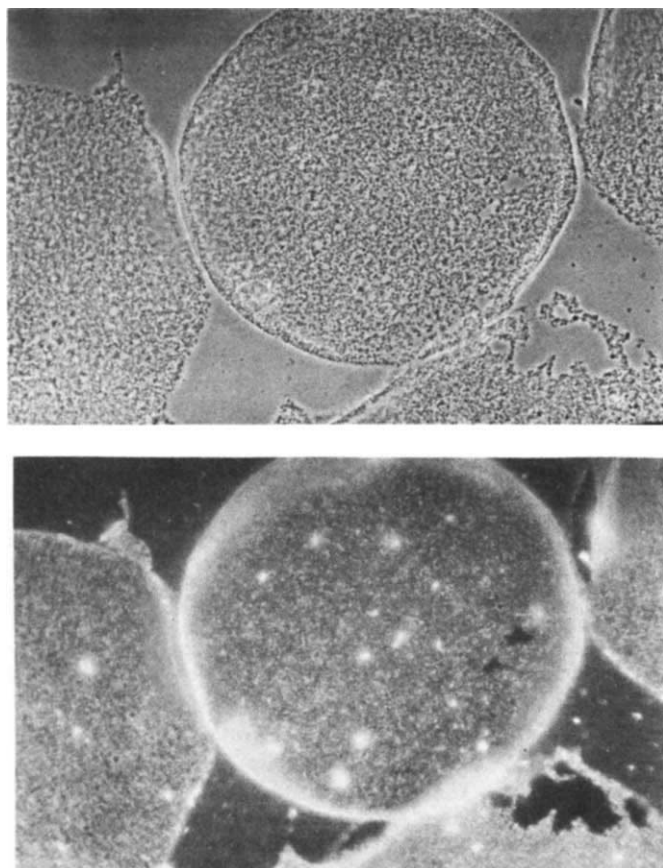


Fig. 2 Indirect immunofluorescent localization of calmodulin on the cytoplasmic side of isolated cortical fragments. Isolated cortical surfaces with only the cytoplasmic side exposed to medium were prepared as described in Fig. 1 legend on poly-lysine-coated glass coverslips. After following the same incubation procedure for 75 min with calmodulin antibody, the cortices were incubated for a further 60 min with fluorescein isothiocyanate-conjugated anti-sheep antibody prepared in rabbit, diluted 16-fold in the isolation medium (Miles). In controls in which the preincubation was with preimmune serum, calmodulin antibody adsorbed with excess calmodulin or isolation medium alone showed little or no staining except for occasional spots where the rabbit fluorescein isothiocyanate-conjugated antibody was trapped in a bit of debris or fold of the cortices. Zeiss epifluorescent microscope, $\times 63$ phase optics. The stuck-down cortical diameter averages 120 μm .

explanation is correct, the delay reflects the time for the lower-affinity components of our heterologous antibody to bind and cover the last sites.

Immunofluorescent localization of calmodulin revealed heavy granular staining in the plane of the plasma membrane just beneath the layer of cortical granules (Fig. 2). During the repeated washes of the immunofluorescent procedure many of the cortical granules were washed away but staining was still found where they had been. Of course we cannot state definitely whether or not calmodulin was associated with the granules as well as the membrane, but it seems most likely that it is associated with both at the region of attachment. Having examined more than 24 samples, we suspect that the pattern of calmodulin staining corresponds to the cortical granule sites since the numbers are consistent with such a logical association; however, this conclusion cannot be convincingly reached using a light microscope and proof would require examination with anti-sheep ferritin-conjugated antibody using an electron microscope.

An important question arises as to whether the isolated fragments can be used as a model for real exocytosis or whether the *in vitro* system operates by some other means of granule discharge. Decker and Lennarz showed that in isolated fragments, a normal vitelline complex with the correct orientation was formed when cortical fragments were exposed to calcium ions¹³. Similar results have been obtained for stuck-down corti-

cal fragments which, when exposed to 3 μM Ca^{2+} , undergo exocytosis, forming the perivitelline space and vitelline membrane (M. J. Whitaker and P. F. Baker, personal communication). Estimates of the peak calcium ion transient at fertilization from aequorin measurements fall in the same range (2.5–4.5 μM)¹. These results and the identical sensitivity to calcium ions for both intact eggs suddenly lysed in Ca^{2+} -EGTA buffers and stuck-down cortical fragments strongly imply that identical mechanisms of exocytosis are involved⁵.

To our knowledge, the results reported here are the strongest evidence to date for direct involvement of a known protein in fusion of a natural biological membrane and exocytosis at physiological concentrations of calcium ions. Our finding implies that calmodulin is bound to the inside surface of the plasma membrane and is integrally involved in the exocytosis of cortical granules, conferring the high sensitivity of calcium on that process. As other exocytotic systems also exhibit a similar dependence on calcium^{3,4}, we expect that calmodulin or other similar calcium-binding modulatory proteins will be shown to be involved in exocytosis generally.

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Measurement of changes in cytoplasmic free Ca^{2+} in fused cell hybrids

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The investigation of many problems in cell biology requires a method for studying chemical 'events' in living cells. For example, the measurement of cytosolic free Ca^{2+} , using Ca^{2+} indicators in giant cells, has provided vital evidence for the role of this ion in the activation of cells by electrical and chemical stimuli¹. However, the incorporation of Ca^{2+} indicators into small cells (diameter $< 20 \mu\text{m}$), without causing irreversible damage, has proved extremely difficult¹. Recently, an elegant new technique has been reported² for entrapping within small cells a fluorescent Ca^{2+} indicator after hydrolysis within the cell of a membrane-permeant ester of the compound. We have developed a method, using Sendai virus, for fusing cells with erythrocyte ghosts containing photoprotein Ca^{2+} indicators and other membrane-impermeant chemiluminescent-labelled indicators^{3,4}. Because of the sensitivity of chemiluminescent analysis, these indicators can be used at concentrations which do not significantly disturb the chemistry of the cells. The ultimate aim was to establish a cell system in which free Ca^{2+} , oxygen radicals and cyclic nucleotides could be directly monitored. Here we report the incorporation of the photoprotein, obelin⁵, an indicator of free Ca^{2+} , into small mammalian cell hybrids.

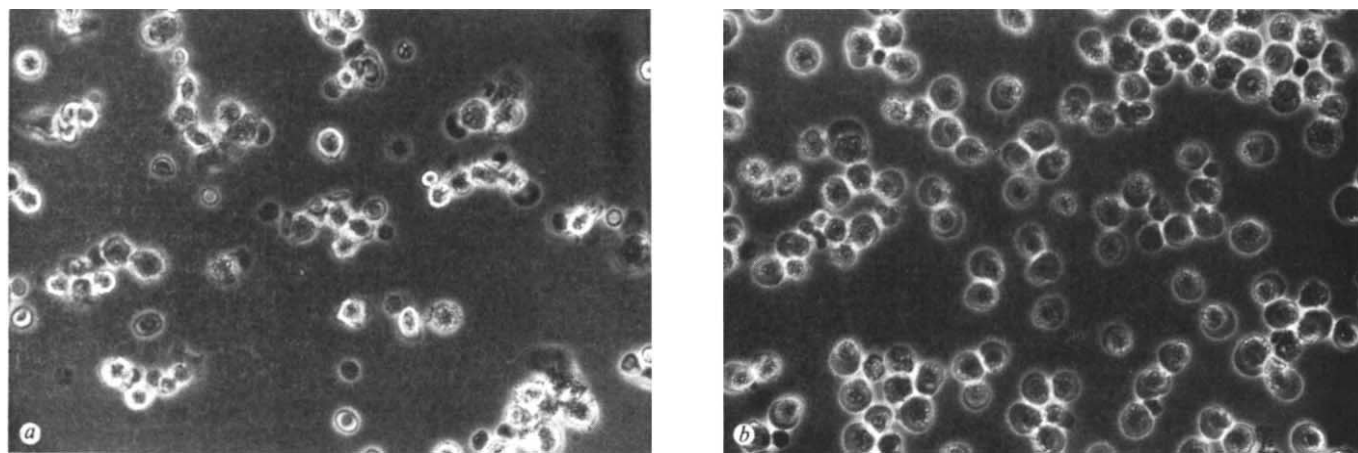


Fig. 1 Phase-contrast microscopy of unfused cells and hybrids. Human erythrocyte ghosts containing obelin were prepared from washed human erythrocytes by lysing preswollen erythrocytes⁶ in 10 mM TES (*N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid, treated with Chelex-100, see ref. 1) and adding obelin (1–10 μ l) for 10 min at 0 °C. Isotonicity was restored by addition of KCl (pure) to give a final concentration of 150 mM, and the ghosts were resealed at 37 °C for 30 min. Rat polymorphonuclear leukocytes were isolated from the peritoneal fluid of rats previously injected intraperitoneally with sodium caseinate as previously described⁷. Fusion was achieved by resuspending a mixture of erythrocyte ghosts (5×10^7) and polymorphonuclear leukocytes (5×10^7) in 100 μ l of ice-cold medium containing 150 mM NaCl (pure), 10 mM TES–Chelex, 0.25 mM uranyl acetate and 1,500 haemagglutinating units of UV-inactivated Sendai virus, pH 7.4. After 10 min at 0 °C, the suspension was incubated at 37 °C for a further 30 min, washed and resuspended in 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 and 25 mM HEPES, pH 7.4. Unfused polymorphonuclear leukocytes were separated from the hybrids by centrifugation through a preformed Percoll gradient (density 1.05–1.09 g ml^{-1}) at 1,000g for 20 min. *a*, Unfused mixture of human erythrocyte ghosts and rat polymorphonuclear leukocytes; *b*, hybrid cells, showing the granules associated with the polymorphonuclear leukocyte portion of the hybrid and the clear cytoplasm associated with the ghosts. Cells and hybrids were stored for 24 h at 4 °C before the photographs were taken.

Human erythrocyte ghosts containing obelin were prepared by a modification of the 'pre-swell' method of Rechsteiner⁶. Rat polymorphonuclear leukocytes were prepared after intraperitoneal injection of sodium caseinate⁷. Fusion was achieved by resuspending a mixture of erythrocyte ghosts and polymorphonuclear leukocytes in a medium containing Sendai virus (Fig. 1). More than 99% of cells in the final preparation were hybrids, as observed by light microscopy (Fig. 1). Oxygen radical formation detected by chemiluminescence was used to monitor a Ca^{2+} -dependent response in the hybrids⁸. A major problem associated with using Sendai virus as a fusogen has been that the virus causes cell lysis. A crucial factor in our method is the use of uranyl acetate in the fusion medium⁹, which reduces virus-induced haemolysis of the ghosts from 89% to 2.5%, as measured by haemoglobin release. A second important feature is the absence of extracellular Ca^{2+} during fusion. Although the role of intracellular Ca^{2+} in cell fusion is controversial¹⁰, we have

demonstrated that an increase in intracellular free Ca^{2+} is an early event in ghosts exposed to Sendai virus and occurs before fusion (P. Fuchs, M.B.H. and A.K.C., unpublished observations). This rise in free Ca^{2+} can cause consumption of >90% of the obelin. However, because this large rise in free Ca^{2+} is not essential for fusion, extracellular Ca^{2+} was omitted from the fusion medium so as to maintain the obelin active in the hybrids. The hybrids were capable of responding to a phagocytic stimulus (latex beads), which induces the production of oxygen radicals, detected by luminol-amplified chemiluminescence^{7,8}. The response of the hybrid cells to latex beads was 50–80% of that in the unfused polymorphonuclear leukocytes.

We have developed a method^{11,12} for quantifying the extent of fusion between the erythrocyte ghosts and polymorphonuclear leukocytes using the activation of complement by two antisera, one specific for the human erythrocyte ghosts and the other specific for the rat polymorphonuclear leukocytes. Formation of

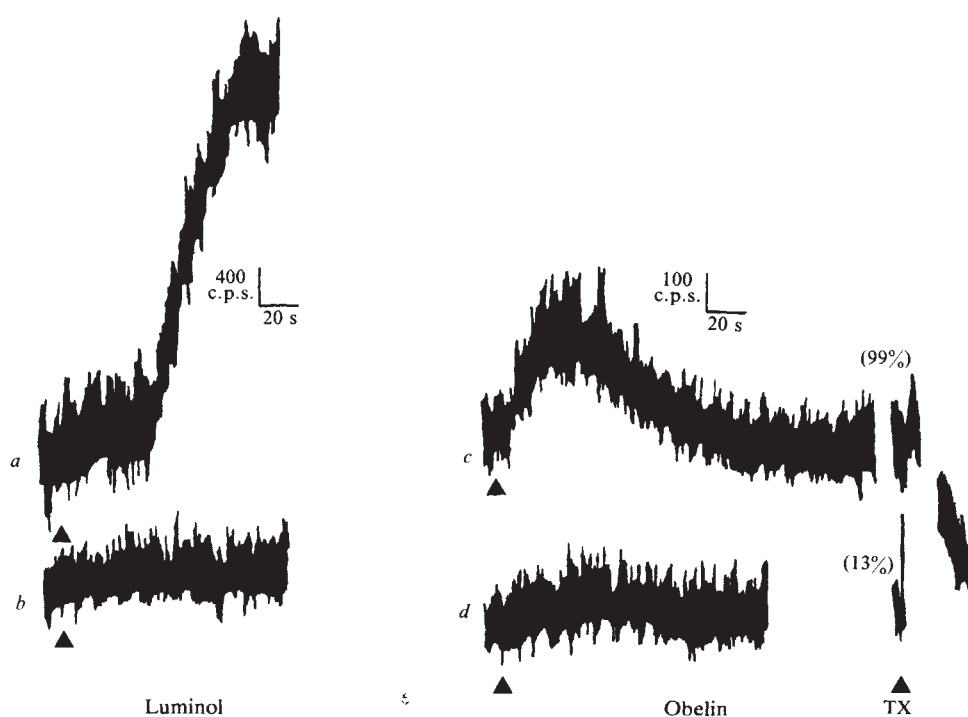


Fig. 2 Obelin luminescence and luminol-amplified chemiluminescence from unfused cells and hybrids. Approximately 5×10^6 hybrids or 10^7 total cells in the unfused mixture were resuspended in 500 μ l of Krebs–Ringer HEPES buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 1.3 mM CaCl_2 , 0.1% bovine serum albumin, (w/v), 25 mM HEPES, pH 7.4) and preincubated for 2 min at 37 °C with either anti-human erythrocyte antisera (20 μ l) (*a*, *b*) or anti-polymorphonuclear antisera (20 μ l anti-rat 5'-nucleotidase antiserum) (*c*, *d*). Oxygen radical formation was monitored after addition of 10 μ M luminol to the extracellular medium by chemiluminescence (*a*, *b*). Obelin luminescence was monitored in the absence of luminol and consumption of obelin was demonstrated by addition of Triton X-100 (scintillation grade 2%, w/v) at the end of the time period (TX), allowing all remaining obelin to be stimulated (*c*, *d*). Values in parentheses show the % obelin consumed. *a*, *c* Show responses from hybrids and *b*, *d* responses for unfused cells. At the arrow, human serum (50 μ l in 500 μ l of Krebs–Ringer HEPES buffer) as a source of complement was added to the suspension. The vertical scale shows luminescent counts per second (c.p.s.) and the horizontal scale shows time.

the complement terminal attack complex on the ghost membrane results in a rapid rise in the intracellular free Ca^{2+} concentration after binding of complement component C9 (ref. 12). Activation of complement by anti-rat polymorphonuclear leukocyte antibody bound to the polymorphonuclear leukocyte surface stimulates oxygen radical formation from the polymorphonuclear leukocyte, and this effect also requires Ca^{2+} and complement component C9. After fusion of the erythrocyte

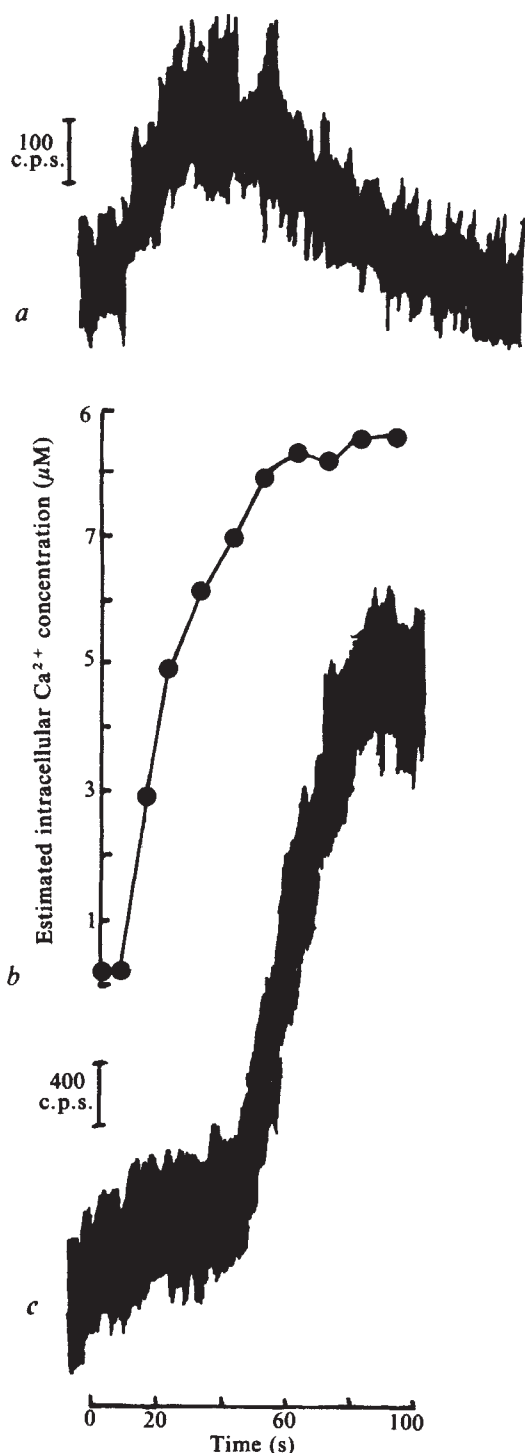


Fig. 3 Relationship between intracellular Ca^{2+} and oxygen radical production from the hybrids. Hybrids were stimulated with anti-polymorphonuclear leukocyte antibody plus complement as described in Fig. 2 legend. *a*, Obelin luminescence; *b*, the cytoplasmic Ca^{2+} estimated from *a*. The rate constant of obelin luminescence was calculated from *a* at various times, and converted to a free Ca^{2+} concentration by reference to a calibration curve relating rate constant to free Ca^{2+} in the presence of 2 mM Mg^{2+} (see ref. 12). *c*, The luminol-amplified chemiluminescence resulting from oxygen radical formation, accompanying stimulation of the hybrids.

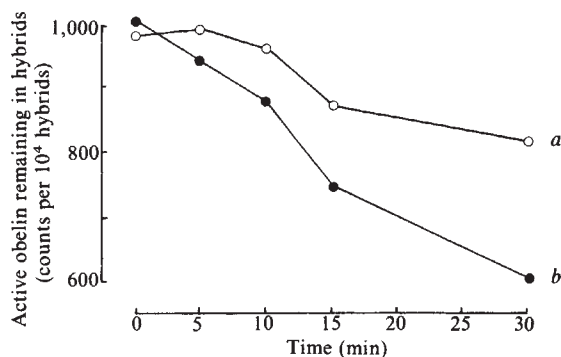


Fig. 4 Consumption of obelin in hybrids stimulated by chemotactic peptide. Total active obelin remaining in the hybrids after various times of incubation was measured by addition of 1% Triton X-100 (scintillation grade) in Krebs-Ringer HEPES buffer to samples of the suspension to stimulate all remaining active obelin. *a*, Incubation at 37°C with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (1 μM dissolved in dimethyl sulphoxide, final concentration 0.1%). *b*, Dimethyl sulphoxide (0.1% v/v) alone. From the curves the mean rate constants of obelin consumption were calculated and compared with calibration curves of obelin luminescence against Ca^{2+} concentration. Assuming the free Mg^{2+} concentration within the hybrid to be ~ 2 mM, the free Ca^{2+} concentration within the hybrids was estimated to be 0.7 μM (*a*) and 0.3 μM (*b*).

ghosts with polymorphonuclear leukocytes, observed microscopically, complement activation by anti-rat polymorphonuclear leukocyte antibody stimulated obelin luminescence and complement activation by anti-human erythrocyte antibody stimulated oxygen radical formation from the hybrids (Fig. 2). In contrast, no obelin response was detectable from the unfused cell mixture when complement was activated by anti-rat polymorphonuclear leukocyte antibody and no oxygen radical formation was detectable when complement was activated by anti-human erythrocyte antibody (Fig. 2). In the conditions described for Fig. 1, 60–80% of the obelin originally entrapped in the ghosts was transferred to the hybrids.

An important application of this fusion technique is the quantification of cytoplasmic free Ca^{2+} in both resting and stimulated cells. The cytoplasmic free Ca^{2+} in the hybrids was estimated by relating the rate constant of obelin to free Ca^{2+} (refs 1, 12). Before fusion, the resting free Ca^{2+} concentration in the ghosts, in the presence of external calcium (1 mM), was ~ 0.5 μM . However, the estimated resting free Ca^{2+} in the hybrids was lower, ~ 0.1 – 0.3 μM assuming an intracellular free Mg^{2+} concentration of 2 mM. Activation of complement by anti-polymorphonuclear leukocyte antibody bound to the hybrid surface resulted in a rise in intracellular free Ca^{2+} which reached a plateau of 8 μM within 1 min (Fig. 3). The initial rise in intracellular free Ca^{2+} preceded that in luminol-dependent chemiluminescence by ~ 40 s. The chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine stimulated oxygen radical formation in the hybrids, detected by luminol-dependent chemiluminescence. It also stimulated a prolonged increase in obelin consumption, the mean free Ca^{2+} concentration over 30 min corresponding to ~ 0.7 μM , twice the resting cytoplasmic Ca^{2+} concentration (Fig. 4). Phagocytic stimulation of the hybrids produced a low level of endogenous luminescence which was not caused by obelin but was the result of oxygen radicals formed within the cells. No obelin luminescence was observed above this endogenous luminescence, and no increased consumption of obelin was observed. We conclude that this phagocytic stimulus did not cause a prolonged rise in intracellular free Ca^{2+} , despite the fact that oxygen radical formation required extracellular Ca^{2+} and was blocked by the calmodulin inhibitor trifluoperazine⁷. The extent of oxygen radical formation caused by various stimuli did not, therefore, correlate with the length of time of the increase in free Ca^{2+} .

The presented method, although limited to cells which will fuse with erythrocyte ghosts, has applications for incorporating other indicators into the cytoplasm of hybrids which are still

capable of responding to a cell stimulus. For example, the chemiluminescent compound luminol, which has been used here extracellularly (Figs 2, 3) to monitor oxygen radicals within the phagosomes, has also been incorporated into the cytoplasm of the hybrids to monitor cytoplasmic oxygen radical formation. Furthermore, macromolecular inhibitors of cell function, such as antibodies, can be incorporated into the hybrids¹³. The uniform distribution of these compounds within our hybrids has been demonstrated by observing fluorescein-

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labelled albumin and immunoglobulin originally entrapped in the ghosts. This method therefore provides cell biologists with an exciting new tool for studying chemical changes in living cells.

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Antibody to hepatitis B surface antigen after a single inoculation of uncoupled synthetic HBsAg peptides

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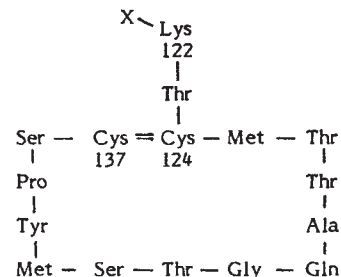
A formalin-inactivated hepatitis B virus (HBV) vaccine has been produced in several laboratories (for review, see ref. 1). The sole source of material for these vaccines has been 22-nm lipoprotein particles composed of hepatitis B surface antigen (HBsAg) and derived from plasma of persons chronically infected with HBV. Such a source presents potential hazards in view of unknown factors that may be present in the plasma, and as high-risk populations are immunized, sources of plasma containing large quantities of HBsAg will become scarce. In addition, no tissue culture system has been developed for the propagation of HBV. The possibility of a synthetic peptide vaccine^{2,3} for HBV had been suggested^{2,3} following studies carried out with tobacco mosaic virus^{4,5} and MS-2 coliphage⁶. This possibility recently became a reality when the amino acid sequence for HBsAg was deduced from the nucleotide sequence of the cloned HBV genome (for a review, see ref. 7). More recently a portion of the major polypeptide derived from HBsAg, with a calculated molecular weight of 25,000 (P25), has been sequenced⁸. Lerner *et al.*⁹ have described a similar approach towards HBV synthetic peptides. Antibody to HBsAg (anti-HBs) was raised in rabbits inoculated with three or four doses of a series of peptides, each containing 14-15 amino acid residues, but only after covalent linkage of the peptides to a carrier protein. Activity was also found after multiple injections of a peptide containing 34 amino acids. We have now synthesized two cyclic peptides containing disulphide bonds, both unrelated to those prepared by Lerner, from a hydrophilic region of the major viral polypeptide, which elicited an antibody response in mice after a single injection without linkage to a protein carrier.

The first major step in our work was the selection of those amino acid residues containing specific HBsAg determinants using a computer analysis of the amino acid sequence of P25 to predict the secondary structure of the molecule according to the technique of Chou and Fasman¹⁰. The protein hydrophobicity was estimated using the amino acid assignments of Bull and Breese¹¹. HBsAg was found to contain predominantly β structure interrupted with a series of β turns. Three highly hydro-

phobic regions are located between residues 1 and 42, 66 and 108, and 146 and 226, and two hydrophilic regions between residues 43 and 65, and 109 and 145. The latter hydrophilic regions are probably exposed on the intact particle and, consequently, β turns in these regions would represent potentially exposed antigenic determinants¹².

The region between amino acids 117 and 137 was chosen for our first synthesis because a cyclic disulphide could be formed between Cys 124 and Cys 137 (see Fig. 1). Thus by locking the conformation of the potential β turns at residues 129-132 and 135-137, we expected to obtain a polypeptide more native in structure⁷. A serine was substituted for the cysteine at position 121 in the *ayw* sequence⁸ to allow formation of a single cyclic disulphide. Lysine was substituted for Arg 122 to provide a handle for cross-linking to other proteins, if needed, before injection into animals. The selected peptides were synthesized by solid-phase methodology¹³ on a Schwarz BioResearch synthesizer modified for computer control¹⁴. Butyloxycarbonyl-S-*p*-methoxybenzyl-L-cysteine was coupled to *p*-hydroxymethyl-phenylacetamidomethyl polystyrene using dicyclohexylcarbodiimide with a catalytic amount of 4-*N,N*-dimethylaminopyridine^{15,16}; the loading was 0.09 mM per g of resin. For the synthesis, the α -amino groups were protected with *t*-butyloxycarbonyl (*t*-BOC) and the side chain-protecting groups were as follows: benzyl ethers for the hydroxyl of serine and threonine, dichlorobenzyl ether for the phenolic hydroxyl of tyrosine and carbobenzoxy for the ϵ -amino group of lysine. Trifluoroacetic acid (40% in CH₂Cl₂) was used to remove the *t*-BOC, and the resulting salt was neutralized with *N,N*-diisopropylethylamine (5% in CH₂Cl₂). Dicyclohexylcarbodiimide was used to couple the *t*-BOC amino acids; hydroxybenzotriazole was added to the coupling reaction of glutamine to

Peptide 1



Peptide 2 contains 5 additional amino acid residues:

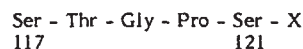


Fig. 1 Amino acid sequences of synthetic peptide 1 (molecular weight 2,219) and peptide 2 (molecular weight 2,739).

suppress side chain dehydration. The specific steps of the synthesizer programme have been published elsewhere^{15,17}.

The protecting groups were removed and the peptide was cleaved from the resin at 0 °C with anhydrous hydrogen fluoride containing 10% anisole and 1% ethanedithiol as scavengers. The HF reagent was removed under vacuum at 0 °C, and then the peptide was precipitated and washed with anhydrous ether. After extraction of the peptide from the resin with trifluoroacetic acid, the solvent was evaporated at 15 °C and the peptide again precipitated with ether. The ether was decanted after centrifugation and the pellet dissolved in 1 M Tris/6 M guanidine hydrochloride; the pH of this solution was immediately adjusted to 8. The solution was desalted on a BioGel P-2 column equilibrated in 0.1 M ammonium bicarbonate, and the peptide-containing fractions were pooled and lyophilized.

The cyclic disulphide was formed by oxidation of a dilute solution of the peptide with potassium ferricyanide according to the procedure of Felix *et al.*¹⁸. The solution was lyophilized and desalted on a BioGel P-2 column, and the peptide further purified by ion-exchange chromatography at 4 °C on a column of SP-Sephadex equilibrated in 0.02 M NaH₂PO₄/6 M urea, pH 3.74. The material eluted with a linear gradient of 800 ml of 0.02 M NaH₂PO₄/6 M urea and 800 ml of 0.15 M NaCl/0.02 M NaH₂PO₄/6 M urea, pH 3.74. The peptide was located by measuring the absorbance at 275 nm.

After desalting on Bio-Gel P-2 in ammonium bicarbonate and lyophilization, we found that each peptide had the expected amino acid composition by amino acid analysis (Table 1) and eluted as a major peak on C₁₈-reverse-phase HPLC in a linear gradient of 1% triethyl ammonium phosphate, pH 3.2, and 2-propanol.

P25 was derived from a purified preparation of HBsAg after solubilization by heating at 100 °C for 2 min in 0.5 M urea/1% SDS/1% 2-mercaptoethanol¹⁹. The soluble polypeptides were fractionated by cylindrical polyacrylamide gel electrophoresis (PAGE) in 10% gels. (P25 was designated P22 in our previous studies^{19,20} based on molecular weight estimations by PAGE.) The SDS-denatured linear P25 polypeptide, which is known to contain 226 amino acid residues, was used as a control for our immunogenicity studies.

The two synthetic peptides (117–137 and 122–137) were incorporated into a series of adjuvant vehicles for immunization of mice. Equal volumes of immunogen and Freund's complete adjuvant (FCA) were emulsified to give a final peptide concentration of 50 µg per 0.4 ml. The alum-absorbed preparations were prepared as described previously²⁰ with hydrated aluminium potassium sulphate, to give a final suspension containing 50 µg peptide per 0.4 ml. Large multilamellar liposomes were prepared from a mixture of dipalmitoylphosphatidylcholine, cholesterol and dipalmitoylphosphatidic acid in a molar ratio of 2.0:1.5:0.2 respectively²⁰. Dried lipid films were dispersed by vortexing in an aqueous medium at a phospholipid concentration of 20 mM. Liposomes were swollen in solutions containing either 100 µg of peptide per ml or 100 µg of peptide plus 1 mg ml⁻¹ muramyl dipeptide (MDP; Sigma). To ensure that comparable doses were given in each case, untrapped antigen was not removed. Previous studies have indicated that ~60% of HBsAg materials were associated with liposomes prepared in these conditions²⁰. Final liposome concentrations were adjusted such that each animal received 6 mmol phospholipid and 25 µg peptide with or without 250 µg MDP in a volume of 0.3 ml.

Groups of six female BALB/c mice were immunized intraperitoneally with each of the above preparations. The mice were bled from the tail vein on days 7, 14 and 21. Samples were assayed at a serum dilution of 1:4 using a commercial solid-phase radioimmunoassay (Ausab, Abbott, Chicago). Anti-HBs concentrations are expressed as milli-International Units per millilitre (mIU ml⁻¹) based on the international reference preparation of anti-HBs (lot 26.1.77) provided by the International Laboratory for Biological Standards, Central Laboratory of the Netherlands Red Cross Transfusion Service. A computer nonlinear regression program, BMDP3R²¹, was used to

Table 1 Amino acid composition of synthetic peptides

Amino acid	Peptide 122–137	Peptide 117–137 Ser 121
Threonine*	3.75(4)	4.67(5)
Serine*	1.93(2)	3.55(4)
Glutamic acid	1.06(1)	1.06(1)
Proline	0.85(1)	1.92(2)
Glycine	1.00(1)	2.00(2)
Alanine	0.99(1)	1.08(1)
Half cystine†	— (2)	— (2)
Methionine	1.87(2)	1.87(2)
Tyrosine	0.99(1)	0.93(1)
Lysine	0.93(1)	0.92(1)

Analyses were performed on a Beckman model 121 amino acid analyser after hydrolysis *in vacuo* for 24 h at 110 °C in 6 M HCl. The theoretical values are given in parentheses.

* Values are not corrected for destruction during hydrolysis.

† Half cystine was present in the amino acid analysis but was not integrated.

describe the standard dose-response curve for the WHO international reference preparation from which the potency of each of the samples was obtained²².

The results given in Table 2 show that (1) approximately 50% of the mice in each group produced detectable levels of antibody 7 days after inoculation of either the synthetic peptides or the P25 polypeptide; (2) the adjuvant vehicle—FCA, alum, liposomes, or liposomes with incorporation of MDP—did not significantly affect the primary antibody response; (3) in several groups the antibody response was greater on day 14, as shown by either the increased anti-HBs concentrations or the number of mice in each group which responded; (4) on day 21, the peak levels of antibody response in most groups of mice decreased; and (5) the antibody response in mice inoculated with purified SDS-denatured P25 was similar to that observed with the synthetic peptides.

When sera from each group of positive mice on days 7, 14 and 21, respectively, were pooled and fractionated by ultracentrifugation in 10–40% (w/v) preformed sucrose gradients²³, IgG antibody was localized in the intermediate fractions and IgM was detected in the rapidly sedimenting fractions. Typical IgM and IgG responses were seen in each group, with one exception: there was no IgG in the group of mice injected with peptide 122–137 emulsified with FCA.

Early work with a plant virus model using tobacco mosaic virus^{4,5} and the bacterial virus model using MS-2 coliphage⁶ clearly demonstrated the immense potential of preparing immunologically active synthetic peptide analogous to amino acid sequences in an animal virus protein. The present results indicate that amino acid residues localized in the major HBsAg polypeptide, P25, between residues 117 and 137—or more specifically, 122 and 137—contain a determinant that elicits the production of antibody of similar specificity to that produced by immunization with the SDS-denatured linear virus P25 polypeptide¹⁹. These findings are similar to those reported recently by Lerner *et al.*⁹, who prepared a series of synthetic peptides containing segments of the P25 HBsAg polypeptide. Four of their peptides (2–16, 22–35, 48–81 and 95–109) elicited the production of antibody in rabbits which reacted with HBsAg. Synthetic peptides of residues 134–146 and 138–149 (refs 24 and 25 respectively) of P25 have also been prepared. These peptides, which have several amino acids (138–146) in common, were both antigenically active in that each reacted with preparations containing specific anti-HBs activity and they blocked antibody reactivity of sera of animals immunized with intact HBsAg. There have been no reports regarding their immunogenic activity. The amino acid sequences of the six unique immunogenic synthetic peptides included in our study and the above studies have nothing in common except the similarity noted above (residues 138–146) and the overlapping of four carboxyl amino acids of the peptides synthesized in our labora-

Table 2 Anti-HBs concentrations in mice immunized with synthetic HBsAg peptides 117-137 and 122-137

Immunizing preparation	Range of antibody concentrations postinjection (mIU ml ⁻¹)		
	Day 7	Day 14	Day 21
117-137-FCA	3.5-8.6(4/6)	3.2-10.3(5/6)	2.6-13.9(5/6)
122-137-FCA	3.6-5.8(3/6)	5.9-11.9(3/6)	17.3-25.9(3/6)
117-137-alum	3.0-7.3(2/6)	2.8-12.6(3/6)	3.4-4.5(2/6)
122-137-alum	2.1-14.5(4/6)	2.5-10.0(3/6)	5.0-6.4(2/6)
117-137-liposomes	2.1-15.4(3/6)	2.0-12.8(4/6)	—
122-137-liposomes	3.6-8.8(3/6)	5.2-12.0(3/6)	5.6-11.8(3/6)
122-137-liposomes +MDP	2.1-23.7(4/6)	2.9-21.0(3/6)	6.8-40.0(2/6)
P25 polypeptide-alum	2.1-17.2(6/6)	2.5-10.5(5/6)	7.1-12.5(2/6)

Only positive antibody values are listed. Numbers in parentheses represent number of mice with an anti-HBs response over total number of mice in each group.

tory with the initial four residues at the amino terminus of the peptide synthesized by Vyas²⁴. Thus a number of different amino acid sequences can elicit production of antibody which specifically reacts with HBsAg. It will be essential to immunize susceptible chimpanzees with several of these peptides to ascertain which elicit(s) the production of protective antibody, as we have done with the purified P25 polypeptide¹⁹. In addition, it will be of interest to learn which sequences contain the cross-reacting 'a' antigenic determinant and which contain the subtype-specific determinants. We selected residues 117-135 because this region is highly hydrophilic, and shows great variation among the published sequences and therefore may contain the subtype-specific determinants^{8,24}. It may be found necessary to use more than one of these peptides to produce a highly effective vaccine.

Note that Lerner *et al.*⁹ elicited an antibody response to their smaller (14-15 residues) peptides only after repeated inoculation of covalently attached material to a carrier protein. In our study, we observed an antibody response to each peptide (117-137 or 122-137) without using a carrier protein. The greater immunogenicity of our peptides, shown by the detection of antibody activity 7 days after a single injection of peptide, may be because our peptides, consisting of 16 or 21 amino acid residues, contain a determinant which is more immunogenic. Alternatively, disulphide cyclization may be important in locking the secondary structure of a potent immunogen.

The incorporation of various adjuvant vehicles and/or protein carriers should help to increase the immunogenicity of these synthetic peptides. This approach may offer advantages in the production of a vaccine against the hepatitis B virus, since the relative immunogenicity of the HBsAg-derived polypeptides is considerably less than that observed for intact HBsAg 22-nm particles¹⁹.

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New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma

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The P3J-HR-1 (HR-1) line contains unique Epstein-Barr viruses (EBVs) which fail to immortalize lymphocytes or to induce lymphomas¹⁻⁴. Virus from Jijoye, the parent of the HR-1 clone, retains lymphocyte-immortalizing ability^{5,6}. Because the HR-1 virus population is a mixture, it has not been possible to identify conclusively the genomic alterations responsible for the nontransforming phenotype⁷. Submolar fragments produced by restriction enzyme digestion and the variety of patterns found after partial denaturation are evidence for heterogeneity in HR-1 virion DNA^{8,9}. To determine whether every cell carried this virus mixture, or whether the line consisted of cells carrying different variants, we separated the mixture by cell subcloning into new variants, which are found to be distinct from parental HR-1 virus both biologically and in genome structure.

HR-1 cells were cloned in agarose with an efficiency of 37% (refs 10, 11). The copy number of EBV genomes in 29 subclones derived in two experiments (FF452, GG68) was determined by nucleic acid spot hybridization¹² (Fig. 1a). The parental HR-1 line contained ~300 genomes per cell; all subclones had lower copy numbers, between 3 and 22 genomes per cell. Following treatment with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) which induces viral DNA synthesis in the HR-1 line¹³, there was an increase, to a minimum of 200 EBV DNA copies per cell, in each of the clones (Fig. 1b). However the response to TPA was exaggerated in five clones (GG68 nos 5, 11, 13, 17 and 19) which after treatment contained 3,000-6,000 EBV DNA copies per cell. These clones are called 'superinducible'. The same five clones released large amounts of viral DNA (4×10^7 - 4×10^8 genomes per ml) into the culture supernatants after treatment with TPA (Fig. 1c). Differences in content of viral DNA correlated with levels of viral capsid antigen (VCA). In all clones antigen expression ($\leq 1\%$) was markedly below the 10% antigen-positive cells found in the HR-1 line. Following treatment with TPA, 50-80% of cells in the five superinducible clones became antigen positive; only slight enhancement of VCA expression was seen in the rest of the clones.

These data indicated heterogeneity among subclones in the extent of viral replication. None of the 29 clones spontaneously released EBV in amounts comparable with the parental line. Cloning of the spontaneous producer may require less stringent conditions. Spontaneous viral particle synthesis in the HR-1 line may involve interaction between different viruses in the line. The superinducible clones will be useful in further studies: as some yield 10-20 times more viral DNA than the parental HR-1 line, they may help in analysis of genome structure and repli-

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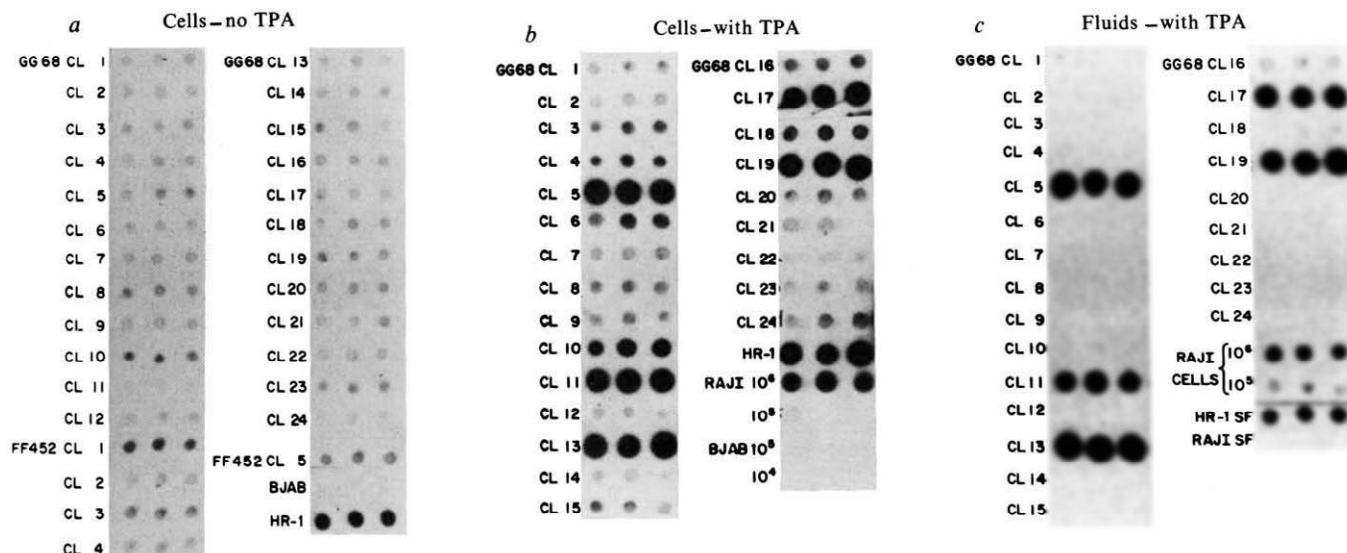
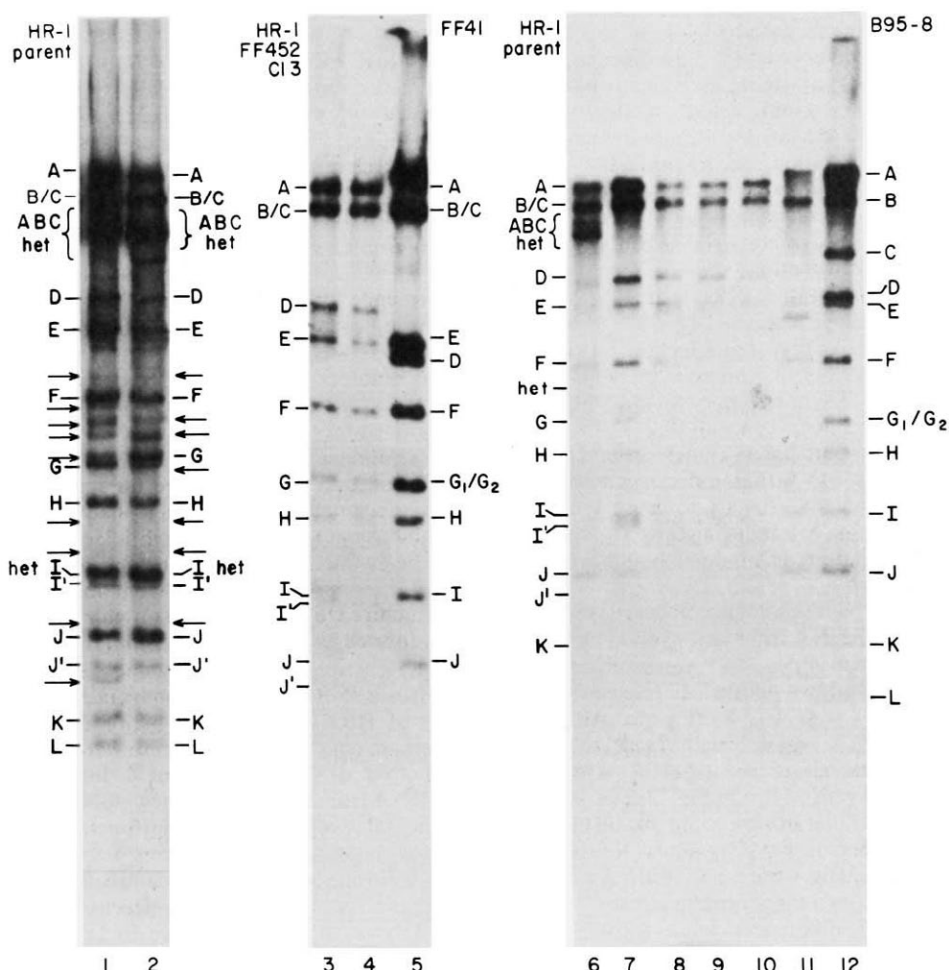


Fig. 1 Estimation of EBV DNA content of the HR-1 line and its subclones by nucleic acid spot hybridization. Each filter was probed with $1-5 \times 10^6$ c.p.m. of nick-translated ^{32}P -labelled EBV DNA prepared with 300 ng of HR-1 DNA. *a*, Genome content of 29 subclones and the HR-1 parent without TPA treatment. Each spot contains 10^5 cells collected 6 days after subculture. BJAB cells (EBV genome negative) serve as negative control. Autoradiograph exposed for 18 h. *b*, Genome content of 24 subclones and the HR-1 parent with TPA treatment. Each spot contains 10^5 cells collected 6 days after subculture with 20 ng ml^{-1} TPA. The positive control was 10^6 and 10^5 Raji cells; the negative control was 10^5 and 10^4 BJAB cells. Neither BJAB nor Raji was treated with TPA. *c*, Genome content of supernatant fluids from 24 subclones and the HR-1 parent collected 14 days after subculture in the presence of 20 ng ml^{-1} TPA. 10^6 and 10^5 Raji cells (no TPA treatment) served as positive control. Raji supernatant fluid (SF) which does not contain virions was negative control. High levels of hybridization seen with fluids in *c* are seen in *b* with intracellular EBV DNA from the same cell clones. Autoradiograph exposure in *b* and *c* was 4 h.

Fig. 2 *Eco*RI digestion of EBV DNA prepared from virions. DNA was extracted from virions shed into 20 l of culture supernatants 14 days after addition of 20 ng ml^{-1} TPA. Lanes 1-5 contained 600 ng of EBV DNA; lanes 6-12 50 ng of DNA. Each panel is an autoradiograph of a Southern blot hybridized with 10^7 c.p.m. of nick-translated ^{32}P -labelled HR-1 DNA. Lane 1, HR-1 parental (LS strain from M. Nonoyama); 2, HR-1 parent (laboratory strain); 3, 4, FF452, clone 3, a 'moderately inducible' HR-1 clone; 5, FF41, a transforming EBV originally from saliva¹⁶; 6, HR-1 parent (LS strain); 7, GG68 clone 5 'superinducible'; 8, GG68 clone 13 'superinducible'; 9, FF452 clone 3 (this DNA was prepared from the clone passaged for 10 months after preparation of the DNA shown in lanes 3 and 4); 10, FF452 clone 5 moderately inducible; 11, FF41; 12, B95-8, a transforming EBV originally from blood. Note that the bands 'ABC het' and other 'het' bands, indicated by an arrow in the DNA of the HR-1 parent, are not seen in the DNA prepared from the cell clones, and that the *Eco*RI A fragment is smaller in the DNA of the parental HR-1 and all of its subclones than in the DNA of the transforming B95-8 and FF41 strains.



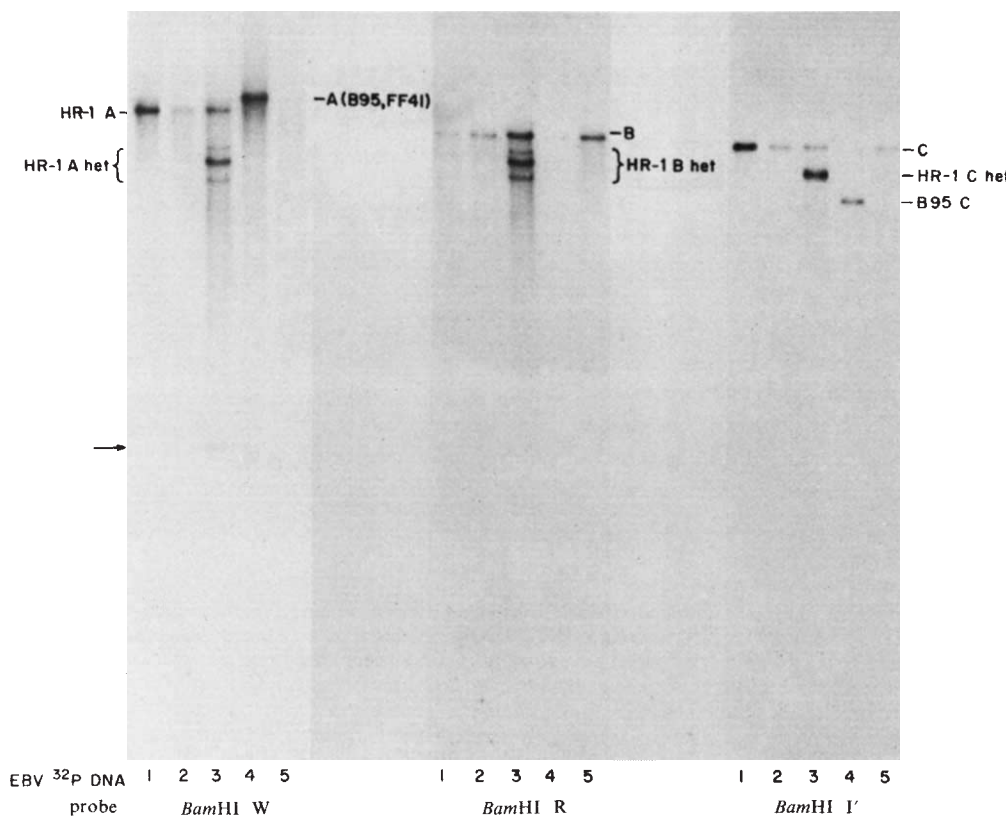


Fig. 3 Hybridization of Southern blots, containing *EcoRI* digests of EBV DNA, prepared from parental HR-1 DNA, two of its cellular subclones and two transforming viruses, with individual *Bam*HI DNA fragments. The latter, derived from strain FF41, were propagated on recombinant pBR322 plasmids¹⁶ labelled with ³²P by nick translation. Lane 1, GG68 clone 13; 2, FF452 clone 3; 3, parental HR-1 (lab.); 4, B95-8; 5, FF41. In *a* the probe was *Bam*HI W, in *b* *Bam*HI R, and in *c* *Bam*HI I'. In each panel note that the probe identifies a group of fragments (A, B and C het) present in the parental HR-1 DNA and absent in the HR-1 cell subclones and the transforming viruses. In *a* the *Bam*HI W fragment faintly identifies an *Eco*RI fragment (arrow) of approximate molecular weight 2.4×10^6 in all strains. The genome location of this fragment is not known. *c* Shows the previously described^{16,22} deletion in the *Eco*RI C fragment of B95-8.

cation. Studies of transcription of the EBV genome in the absence of productive infection and after TPA induction should ultimately clarify mechanisms by which the cell-virus relationship changes from latency to virus production.

We next analysed biological properties of subclonal virus. Undiluted supernatant fluid from all clones was cytotoxic and failed to immortalize human neonatal lymphocytes; furthermore, transforming activity could not be demonstrated in five clones (FF452 nos 1-5) tested by the more sensitive method of X-ray irradiation and co-cultivation¹⁴. Virus from two clones interfered with lymphocyte transformation by the B95-8 strain of EBV. Subclonal viruses thus resembled the parental HR-1 strain in lack of immortalization, cytotoxicity and interference with transformation.

Unconcentrated HR-1 virus, grown in the presence or absence of TPA, caused 15-30% of Raji cells to develop early antigen (EA) 72 h after infection¹⁵. In contrast EA synthesis was not stimulated in Raji cells infected by clonal virus obtained without TPA, or by a pool of supernatants from all 29 subclones which had been concentrated 35-fold. Although virus stocks from superinducible clones after TPA treatment contained more viral DNA than did stocks of parental HR-1 virus, they still lacked the capacity to cause EA synthesis. As subclonal virus lacking EA-inducing capacity is cytotoxic and inhibitory to transformation, EA induction and cytotoxicity must be distinct functions.

To correlate biological properties with genome structure we examined DNA from virions shed by the HR-1 line, four of its cellular subclones (two superinducible, two moderately inducible) and two lines which release immortalizing virus B95-8 and FF41 (refs 16-18) (Fig. 2). Among fragments of HR-1 virion DNA digested with *Eco*RI were many heterogeneous bands (the larger are labelled 'ABC het' and the rest are indicated with arrows in Fig. 2). Two stocks of HR-1 DNA from different laboratories could be distinguished by the relative prominence of 'het' fragments. Neither 'ABC het' nor smaller 'het' fragments were found in DNA of the four HR-1 subclones nor in the two transforming viruses.

Several differences were found between the DNAs of nontransforming viruses (parental HR-1 and its subclones) and

the two transforming strains (Fig. 2). These will be described fully elsewhere (M.R. and G.M., in preparation) but briefly: *Eco*RI A is consistently larger in both transforming viruses than in the four nontransforming HR-1 subclones, as has previously been shown for the HR-1 parent¹⁹⁻²¹, due to a 1.95 megadalton deletion of *Bam*HI Y and part of *Bam*HI H. In HR-1 and its clones, *Eco*RI G₂ is replaced by two new fragments, *Eco*RI I' and J' (ref. 21). *Eco*RI D, the right terminus, is larger in HR-1 than in either transforming virus. In the HR-1 clones there is loss of one restriction site between *Bam*HI B and G and another between *Bam*HI W' and I'. There is also a deletion in *Bam*HI L ($\sim 5 \times 10^4$ daltons) and an insertion in *Bam*HI P ($\sim 6 \times 10^4$ daltons) in the nontransforming virus. Thus differences between transforming and nontransforming variants are scattered over at least six regions of the genome; however there is extensive homology and general similarity in genome organization between transforming and nontransforming EBVs^{20,21}. It remains to be determined which lesions found in the genomes of HR-1 clones are also present in the Jijoye line which retains the transforming component.

Absence of ABC het and smaller het fragments from the *Eco*RI digestion pattern of subclonal viral DNA suggested that heterogeneity had been eliminated by cell cloning. To prove this point we hybridized Southern blots, containing *Eco*RI digests of HR-1, subclone and transforming virion DNA, with probes prepared from individual *Bam*HI fragments of EBV DNA contained on chimaeric plasmids¹⁶ (Fig. 3). The probes were fragments *Bam*HI W, R and I' which are found, respectively, in *Eco*RI fragments A, B and C^{17,18}. Virion DNA from two HR-1 cell subclones and from two transforming viruses each contained only one major fragment with homology to the probes. In contrast there were at least three or four size variants of *Eco*RI 'A', 'B' and 'C' in parental HR-1 DNA.

These new subclonal mutant EB viruses will be useful in deciphering functions of the EBV genome. They could allow unambiguous mapping of the transforming region and should also help identify the genes responsible for EA induction in Raji cells. An attractive hypothesis is that EA induction by HR-1 virus is due to DNA represented by one or more ABC het fragments which were eliminated by cellular cloning.

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Evidence from mtDNA sequences that common laboratory strains of inbred mice are descended from a single female

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The rapid evolution¹ and maternal mode of inheritance² of mitochondrial DNA (mtDNA) make it a valuable marker for individuals related by descent from the same female³. We report here a study of wild and inbred mice which illustrates this point. We have examined mouse mtDNA from various locales in the Northern Hemisphere using restriction enzymes that cut the molecule at an average of 150 sites and find a high level of restriction-site polymorphism in wild mice but no variation among the 'old' inbred strains. This result is surprising because the nuclear genomes of these strains are known to differ greatly from one another⁴. The old inbred type of mtDNA occurs rarely in wild mice and differs from other types in the wild by an average of 100 nucleotide substitutions. In possible contradiction of published breeding records, these findings indicate that only one female lineage contributed to the formation of all the old inbred lines. In addition, our study of new inbred strains provides genetic markers that will be useful in mouse developmental and cell biology.

Thirty-four samples of *Mus domesticus* mtDNA were obtained from 14 inbred strains and 20 wild mice collected in North America, western Europe, North Africa and the Near East. (The name *Mus domesticus* has been coined for a newly recognized species that includes the common laboratory strains and the wild mice populations formerly classified as the sub-

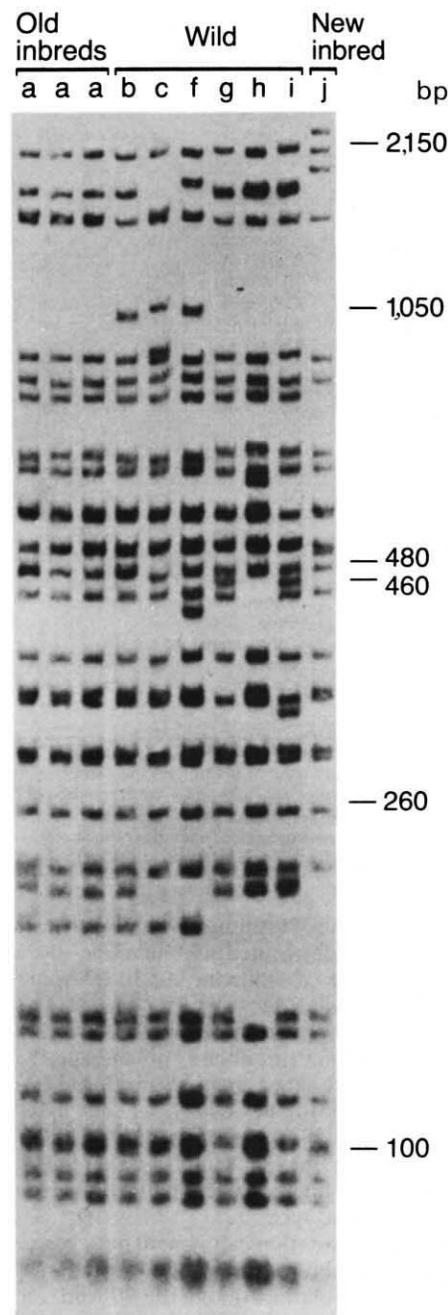


Fig. 1 Electrophoresis of 10 mouse mtDNAs. After purifying mtDNA from tissues of single mice and digesting it with *MboI*, the fragments were labelled and separated by electrophoresis as described elsewhere²⁷. Lanes 1-3 show the identity of fragment patterns for three old inbred mice, C57BL, BALB/c and SWR. Lanes 4-9 show variation among six wild mice whose fragment patterns correspond to those indicated by small letters in Table 1. The animals are from diverse geographical areas: Maryland (USA), Egypt, Switzerland, Morocco, Yugoslavia and Denmark. Lane 10 shows the fragment pattern of a new inbred strain, SF/Cam, founded from a wild mouse in California. The sizes in base pairs (bp) of the size standards (PM2 phage DNA digested with *HindIII*) are indicated on the right.

species *M. musculus domesticus*, *M. musculus brevisrostris* and *M. musculus praetextus*⁵. This species differs substantially from *M. musculus*, the house mouse of eastern Europe (formerly known as the subspecies *M. musculus musculus*), in anatomy, behaviour and protein structure^{5,6}, as well as in mtDNA structure⁷. Moreover, the two species are almost completely isolated from each other genetically due to geographical and multiple sterility barriers⁸). The sources of the 34 mtDNA samples are

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Table 1 Fragment patterns of mitochondrial DNA found in wild and inbred mice

Origin													Differences from inbred strain		
Sample no.	Strain or locality	State or country	Fragment pattern										Fragment patterns	Point mutations	
			<i>Fnu</i> II	<i>Hae</i> III	<i>Hinf</i> I	<i>Hpa</i> II	<i>Mbo</i> I	<i>Taq</i> I	<i>Ava</i> II	<i>Acc</i> I	<i>Hinc</i> II	<i>Hind</i> III			<i>Xba</i> I
Wild mice															
1	Buellton	California	a	a	a	a	a	a	a	a	a	a	a	0	0
2	Vejrumbo	Denmark*	a	a	a	a	a	a	a	a	a	a	a	0	0
3	San Pablo 1	California	a	a	b	a	a	a	b	a	a	a	a	2	23
4	Centerville	Maryland	a	a	a	a	b	a	c	a	a	a	a	2	28
5	San Pablo 2	California	a	a	b	b	a	a	b	a	a	a	a	3	41
6-7	Napa, Orinda	California	a	a	c	a	a	b	a	a	a	b	a	3	66
8	Faiyum	Egypt	a	a	d	a	c	c	c	a	a	b	a	5	198
9	Giza	Egypt	a	a	e	a	d	d	c	a	a	b	a	5	165
10	Erfoud	Morocco	a	a	f	c	e	e	a	a	a	b	a	5	116
11	Viborg	Denmark*	a	b	g	a	a	f	a	b	b	a	a	5	132
12	Petaluma	California	b	a	h	a	c	d	a	a	a	b	a	5	182
13-15	Evansville	Indiana	b	a	h	a	c	d	a	a	a	b	a	5	182
16	Nyon	Switzerland	a	c	i	a	f	d	a	a	c	b	a	6	182
17	Jerusalem	Isreal	a	b	j	a	g	b	c	c	b	b	a	8	182
18	Azrou	Morocco	a	b	j	b	g	g	d	c	b	b	a	9	215
19	Metkovic	Yugoslavia	a	d	k	d	h	b	c	c	b	b	a	9	182
20	Skive	Denmark*	a	b	l	a	i	b	d	b	b	b	b	9	230
Inbred mice															
1-9	Old inbreds	Various	a	a	a	a	a	a	a	a	a	a	a	0	0
10	MOR	Ohio	a	a	a	a	a	a	a	a	a	a	a	0	0
11	PAC	Pennsylvania	a	a	b	a	a	a	a	a	a	a	a	1	12
12	IS/Cam/J	Israel	a	c	m	a	f	d	a	d	a	b	a	6	182
13	SF/Cam/J	California	a	e	n	a	j	h	c	a	b	b	a	7	198
14	SK/Cam/JEi	England	a	e	n	a	j	h	c	a	b	b	a	7	198

Point mutations were estimated by multiplying the number of base pairs (16,295) in mouse mtDNA¹⁰ by the number of base substitutions per base pair (calculated from the fraction of restriction fragments in common by the method of Nei and Li⁹). Old inbreds are BALB/cJ, C57BL/6J, C58/J, DBA/2N, SWR/J, AU/SsJ, C3H, PL/J and AKR/Cu. A cell line (LA9) represented the C3H strain.

* The Danish populations are classified as another species (*M. musculus*) on the basis of morphological and protein evidence⁵, but possess *M. domesticus* DNA, probably owing to introgression⁷. The three populations are close to the zone of contact with *M. domesticus*²⁶.

given in Table 1. Nine of the inbred strains were established over 40 yr ago and are designated as 'old'. The five other inbred strains were established within the last 30 yr⁴ from female house mice caught in the wild.

Highly purified preparations of mtDNA were digested with 11 restriction enzymes, producing, on average, 150 restriction fragments per mouse. The fragments produced by each enzyme were separated electrophoretically, as shown for 10 mice in Fig. 1. We detected 58 fragment patterns; Table 1 shows their distribution among the 34 samples. The mice are ordered according to increasing number of pattern differences from the most common inbred type.

We found much variation in fragment patterns among the wild mice and the new inbred strains but no variation among the old inbred mice (see Table 1). Among the 20 wild mice studied we found 15 distinct types of mtDNA; one type corresponds to that found in the old inbred mice. It was found only twice, once in California and once in Denmark. A more recent survey using the enzymes *Hinf*I and *Mbo*I revealed six more mtDNA types in 36 additional wild mice, none of which had the old inbred type (R.D.S., S.D.F. and A.C.W., unpublished observations). The frequency of occurrence of the old inbred type is therefore 0.04 in the wild mice tested.

The extent to which these DNAs differ in nucleotide sequence from the old inbred type may be estimated from the number of fragments they have in common⁹. These estimates are based on the assumption that the differences in fragment pattern are due exclusively to base substitution. There is evidence in support of the assumption that mouse mtDNA evolves almost exclusively by base substitution^{7,10}. The old inbred type is estimated to differ from other types of *M. domesticus* mtDNA by 12-230 base substitutions (Table 1). The mean difference of 137 substitutions corresponds to almost a 1% sequence difference, which is typical of the mtDNA diversity found in other mammal species¹¹⁻¹⁵. Our result deserves emphasis because earlier surveys using fewer restriction sites^{16,17} and fewer mice¹⁸ seemed to indicate a low level of mtDNA polymorphism in wild mice.

The high level of variation in mtDNA of wild *M. domesticus* is

consistent with electrophoretic^{19,20} and immunological²¹ evidence for a high level of nuclear gene variation in this species. However, the absence of mtDNA variation among the old inbred strains contrasts sharply with the presence of large nuclear differences in these strains⁴. To account for this, we suggest that a single female lineage founded all the old inbred strains, the nuclear diversity among these strains being due mainly to the mating of this lineage with diverse males. This hypothesis agrees with the recorded origins of inbred strains of laboratory mice^{4,22}. The nine old inbred strains listed in Table 1 are thought to stem from a minimum of five different mothers which founded the primary strains as follows: DBA (in 1909 from a random stock at the Bussey Institute, Cambridge, Massachusetts), BALB/c (in 1913 from an Ohio pet dealer), SWR (before 1920 from a random stock at the Pasteur Institute, Paris), PL (before 1922 from a New Jersey pet dealer), and C57-C58 (in 1921 from a Massachusetts pet mouse dealer). If these five females had been picked at random from the wild, the probability of them all having the old inbred type of mtDNA seems, from our survey, to be extremely low ($\sim 10^{-7}$). It is likely, however, that all were from the pet mouse trade^{23,24}, in which case the old inbred type of mtDNA could already have been present at high frequency. A high frequency could be the result of a chance event when the pet mouse population was very small. Such an event could have occurred as long ago as 3,200 BP, when the white mouse cult began in the temple of Apollo Smintheus²⁵.

Selection, rather than chance fixation, could also explain why all the old inbred strains contain the same type of mtDNA and thus appear to be descended from one female. A high frequency of the old inbred type of mtDNA could be attributed to selection for mice bearing this DNA if they were better suited for survival in the pet mouse trade. Selection could act in a second way, by this DNA type giving mice a better chance of surviving laboratory inbreeding. If this were the case, the five primary strains could represent the most successful of many attempts to establish inbred lines. As early publications do not record failures to establish inbred strains, it is uncertain whether such selection

need be invoked. However, a selectionist explanation does not account convincingly for the ease with which wild mice give rise to new inbred strains possessing other types of mtDNA, for example, PAC, IS/Cam and SF/Cam (see Table 1). Furthermore, our finding of the SF/Cam type of mtDNA in wild mice of the San Francisco area (R.D.S., S.D.F. and A.C.W., unpublished observations) is evidence against the argument that this inbred type evolved *de novo* in laboratory conditions. Given that an explanation involving chance fixation could account for the data, we think it unnecessary to invoke an additional factor, such as selection.

Contamination of one strain by another also ought to be considered in relation to the hypothesis that all the old inbred strains trace back to one female in the pet mouse population. The five primary strains could have been crossed soon after they were established in the laboratory, which would imply that published reports are inaccurate in describing the early breeding programmes. It is likely that such a mix-up could have occurred between 1919 and 1921 when Strong and Little had the founding stocks of three of the critical strains (DBA, BALB/c and C57-C58)^{4,22}. Any inter-strain matings at this time, while the DBA strain was at a low ebb, might reasonably link this strain with at least one of the others.

The reason for suggesting this possibility is that the inadvertent mixing of inbred strains may be a common phenomenon even today. There are four recent cases of apparent contamination. (1) Festing²³ has described contamination of the C3H and AKR strains, based on nuclear evidence. (2) There is the case of the new inbred strain MOR, founded from wild mice in Ohio⁴, whose black coat colour is evidence for the presence of nuclear genes from an old inbred strain (probably C57BL/6) in the MOR genome, as this coat colour is rare among wild house mice in North America. Consistent with the idea of contamination, MOR has the old inbred type of mtDNA (see Table 1). (3) Two new inbred strains (SF/Cam and SK/Cam), although founded by wild females in California and England, respectively, have the same rare type of mtDNA (see Table 1). The SK strain examined has probably been mixed with the SF strain because the mtDNA fragment patterns observed for SK/Cam/Ei differ greatly from those reported by Yonekawa *et al.*¹⁷ who used another source of SK/Cam mice. In addition, the SK/Cam/Ei strain carries several protein variants that are diagnostic of the SF/Cam strain (E. Eicher, personal communication). (4) The most spectacular example is provided by two non-inbred strains of the Japanese house mouse, *M. molossinus*^{5,6}. One strain, maintained by Potter at NIH, retains the *molossinus* type of mtDNA, which differs by almost 4 per cent in sequence from that of *M. domesticus*⁷. The other strain, KL/oci, is derived from Potter's strain and has been propagated in a series of other laboratories for >10 yr; KL/oci now has the old inbred type of mtDNA, while retaining proteins characteristic of *M. molossinus*. As extensive sampling of wild mice in Japan revealed no *domesticus*-like types of mtDNA¹⁶, this could be the clearest case of laboratory contamination of a strain by *domesticus* mice bearing the old inbred type of mtDNA.

Thus, we propose that the old inbred mouse strains could be descendants of a single female that lived as recently as 1920, in which case she or her daughters cross-bred with the five primary strains of inbred mice. Alternatively, she could have been a member of the pet mouse population possibly as long ago as 3,200 BP. Besides contributing to knowledge of the genealogy of old inbred mice, our mtDNA study draws attention to cases of contamination of other stocks of laboratory mice and provides new genetic markers for use in cell and developmental biology, for example, markers for cells in chimaeras.

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Recent divergence from a common ancestor of human IFN- α genes

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The two major classes of human type I interferons, leukocyte or α -interferon (IFN- α) and fibroblast or β -interferon (IFN- β) are antigenically distinct and encoded by separate structural genes¹. Recently, genomic hybridization experiments have demonstrated the presence of ~10 or more distinct human IFN- α (Hu IFN- α) genes which code for a family of homologous yet distinct proteins^{2,3}, and 8 of these have been identified and sequenced³. In contrast, no evidence has been found for multiple Hu IFN- β genes³, although heterogeneity of Hu IFN- β mRNA has been observed^{4,5}. The coding sequences of Hu IFN- α and IFN- β genes show weak but appreciable homology, indicating that they were derived from a common ancestor at a remote time⁶, whereas Hu IFN- α_1 (a member of class D) and Hu IFN- α_2 (a member of class A) diverged quite recently (~22 Myr ago)⁷. We report here that all eight known IFN- α genes diverged from a common ancestor quite recently, at most ~26 Myr ago. As mammalian divergence is known to have occurred ~75 Myr ago⁸, this suggests that the IFN- α multigene family in primates is distinct in organization from that of other mammals. We provide evidence for a much lower rate of evolution for IFN- α G than for other α -interferons, and a rapid evolution of IFN- α E pseudogene similar to that of other pseudogenes⁹⁻¹¹. A possible evolutionary origin of size heterogeneity in the 3'-noncoding region of IFN- α mRNAs is also discussed.

A single base change in a codon results in either replacement of the encoded amino acid (replacement substitution) or the appearance of a synonymous codon (synonymous substitution). We shall refer to a site where a single base change leads to synonymous (replacement) substitution as a synonymous (replacement) site¹². At each position in the codon, three

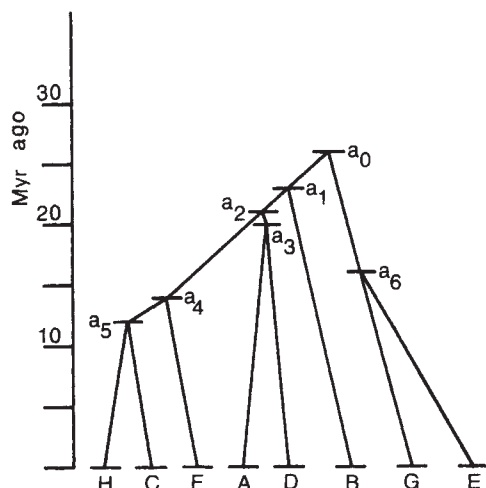


Fig. 1 Evolutionary relationships of eight distinct Hu IFN- α genes. The tree was constructed as follows. Based on the K_S values of pairs of seven functional genes, we first constructed a dendrogram using the clustering method³². This method gives an average value of K_S ($\bar{K}_S$) for all pairs of genes between the groups which are derived from a particular node. For example, $\bar{K}_S$ for pairs between groups (H, C) and group (F), which are joined to each other at node a_4 , is estimated as 0.127 and that for pairs between groups (H, C, F) and (A, D), which are joined at node a_2 , is 0.190, and so on. From $\bar{K}_S$, we have estimated the divergence times of nodes a_0 – a_5 as described in the text. The estimated times are 26, 23, 21, 20, 14 and 12 Myr for a_0 – a_5 , respectively. The date t_6 corresponding to node a_6 was also estimated from K_S as follows. Let O be the common ancestor of G and E; the average difference of synonymous sites between O and G is 0.075 as described in the text. Correcting for multiple hits using $K^c = -(3/4) \ln(1 - (4/3)K)$ ^{16,17}, and dividing the corrected value by $v_s (= 5.1 \times 10^{-9}$ per site per yr), the rate of change at the synonymous sites, gives $t_6 = 16$ Myr.

different single base changes are possible, of which a fraction (f) results in synonymous substitutions, while the remainder $1-f$ leads to replacement substitutions. In the codon UUU, for example, the first two positions are replacement sites ($f=0$) whereas for the last position one-third of changes correspond to synonymous sites ($f=1/3$). Thus the codon UUU contains $0.333(=0+0+1/3)$ synonymous sites and $2.667(=1+1+2/3)$ replacement sites.

Previously, on the basis of this definition, we presented a method for estimating nucleotide differences per site at synonymous sites (K_S) and at replacement sites (K_A) by comparing homologous nucleotide sequences of coding regions¹². Applying the method to several mammalian genes, we showed that the rate of synonymous substitution, v_s , is greater than that of replacement substitution and, more important, is approximately constant among different genes¹³. This is in sharp contrast to the rate of protein evolution, which varies for

different proteins^{8,14,15}. Thus, because the rate of synonymous substitution for different genes is uniform, it can be used as a 'clock' to show the true evolutionary branching orders of genes in a multigene family, even when the encoded proteins evolve at different rates. For example, if two duplicated genes are compared and the K_S determined, then the time (t) since divergence of the two genes can be estimated from the formula^{16,17}

$$t = -(3/4) \ln(1 - (4/3)K_S) / 2v_s \quad (1)$$

using the value of v_s previously calculated as 5.1×10^{-9} per site per yr¹³. Here, K_S is corrected for the effect of multiple hits (a computer simulation showed that this formula is suited for correcting K_S and K_A at least within a short evolutionary period [unpublished results]). Recently, we have confirmed the uniformity of the synonymous substitution rate by comparing many more sequences of different mammalian genes¹⁸.

From pairwise comparisons of coding sequences of eight distinct Hu IFN- α genes, we have calculated K_S and K_A for each pair of genes (Table 1). Based on the K_S values, phylogenetic relationships among the IFN- α genes, and IFN- α pseudogene, were determined (Fig. 1). The seven functional IFN- α genes were classified into three groups according to the divergence times from ancestors. Surprisingly, all eight Hu IFN- α genes seem to have diverged from a common ancestor quite recently, at most ~26 Myr ago. As mammalian divergence is known to have occurred ~75 Myr ago⁸, this result suggests that they have different organization compared with other mammals. This notion is of particular interest in relation to the observation that interferons are often species specific¹⁹. To test this, IFN- α gene sequences have been compared for different species. A pairwise comparison of the N-terminal 20-amino acid sequence of a mouse IFN- α ²⁰ with the six human α -interferons (A–D, F, H) showed that the latter are much more homologous to each other than to the mouse IFN- α . In fact, of 20 amino acid sites compared, on average 3.8 ± 1.9 sites differ within the six human α -interferons, whereas there are 8.8 ± 1.7 site differences between human and mouse α -interferons. Although the comparison was limited to the N-terminal region, this result in part supports the above argument.

The K_A matrix for seven functional IFN- α genes gives almost the same phylogenetic relationship among the genes as that shown in Fig. 1, except for the IFN- α G gene line. Unusually small K_A values were found for gene pairs with the G gene as a partner. Ratios of K_A/K_S for these pairs are lower (0.26 on average) than those for other pairs (0.43), suggesting that the replacement sites of IFN- α G gene have a low evolutionary rate (note that the ratio for a given pair is insensitive to the divergence time of two compared sequences). Alternatively, the IFN- α G gene may have arisen quite recently, as predicted by K_A values, and accumulated more base substitutions at the synonymous sites than other genes. To resolve this, we compared further the sequences of the 3'-noncoding region (which corresponds to a region upstream of the poly(A) site of the A gene in the aligned sequence³) for the seven functional

		50
IFN- α_1 (3'UF)	aatccattatgtgtgtgttcattaaactttactataggaa-cttcctgtat---gtgttcattctttaatat-gaaa	
B (mRNA)	-----ATTTGCCATGTTTATTAATTTTACTATGAAAAATCTT-TAT-----TTATCTTTAAATGAA-	
F (mRNA)	AATATATTATTTT-CTTTT-ATTAATTTTACTATGAAAA-CTTCTTATATTATTGGTTATTCTTTAATAAGAAA	
C (mRNA)	AATATATTAATTT-CCTTTTCATTAAATTTTACTATAC (polyA)	
H (mRNA)	AATATATTAATTT-CCTTTTTCATTAAATTTTACTAT (polyA)	
E (mRNA)	AATATATTACTTTGCTTTTTCATTAAATTTTACTATGG (polyA)	
		100
IFN- α_1 (3'UF)	ttcctagcc--tg-actgtgcaacctgattagagataaagggt	
B (mRNA)	CTCCAA-CCCAGATTGTGCAAACTGATTAAAGAAATGGATGGT (polyA)	
F (mRNA)	TTCCAAGCCC (polyA)	

Fig. 2 Alignment of the untranscribed 3'-flanking (3'UF) sequence of IFN- α_1 (ref. 2) (a member of class D)³ and the terminal sequences of five IFN- α mRNAs³. The sequence immediately after the poly(A) site of IFN- α_1 is indicated by lower case letters. Bases that are identical between the IFN- α_1 and B are indicated by dots. The hexanucleotides AATAAA and ATTAAA are underlined. Broken lines indicate gaps introduced to obtain maximum homology.

Table 1 Sequence differences at synonymous sites (K_S) and at replacement sites (K_A) from pairwise comparison of eight distinct Hu IFN- α gene sequences

		K_S							
		I			II			III	
IFN- α		C	F	H	A	B	D	G	E
K_A	I								
	{ C	—	0.128	0.117	0.202	0.216	0.234	0.236	0.286
	{ F	0.055	—	0.126	0.193	0.193	0.176	0.203	0.256
	{ H	0.055	0.069	—	0.151	0.191	0.186	0.189	0.232
	{ A	0.078	0.078	0.072	—	0.218	0.176	0.227	0.304
	{ B	0.094	0.091	0.075	0.075	—	0.202	0.254	0.265
	{ D	0.079	0.069	0.079	0.072	0.098	—	0.230	0.298
	III	G	0.062	0.046	0.052	0.071	0.057	—	0.200
	E	0.116	0.098	0.098	0.105	0.121	0.109	0.072	—

K_S (upper half) and K_A (lower half) were calculated by a method described elsewhere¹². Only the sequence encoding the C-terminal 133 amino acids is available for the coding region of the IFN- α G gene³. To make equivalent comparisons of all the gene sequences, only differences in this region were considered for all the genes (even when coding sequences corresponding to mature IFN- α are compared, similar values are obtained for K_S and K_A , although in this case, only the fragment-length sequence is considered in pairwise comparisons involving IFN- α G). IFN- α B gene contains frameshift mutations at codons 98 and 101 (ref. 3), thus this segment was excluded from the comparisons. IFN- α E gene contains a single base insertion³; to restore the original reading frame, this insertion was deleted. Functional genes were classified into three groups, I (IFN- α C, F and H), II (A, B and D) and III (G), as the ancestral nodes leading to the present-day genes within each group appear to be in clusters with respect to the divergence time (see Fig. 1).

genes, and obtained a phylogenetic tree (data not shown) essentially identical to that shown in Fig. 1. In addition, the extent of sequence divergence in the noncoding region was similar to K_S in value. These results support the former interpretation.

Using the deduced divergence time, we estimated the rate of evolution at the replacement sites of the G gene (v'_A) and that of other functional genes (v_A). For all pairs between groups which were derived from a particular node in Fig. 1, except for a_0 and a_6 , the K_A values of the pairs were averaged ($\bar{K}_A$) and then the v_A calculated as $v_A = \bar{K}_A / 2t$, where t is the divergence time corresponding to the node and $K^c = -(3/4) \ln [1 - (4/3)K]$. For example, the one containing H and C and the other containing F, were derived from node a_4 , and thus the pair F-H and F-C have the same divergence time ($t = 14$ Myr) corresponding to the node. The average difference $\bar{K}_A$ is 0.062, which gives $v_A = 2.3 \times 10^{-9}$ per site per yr, and so on (Table 1). The average value of v_A , estimated for the five different nodes, is 2.1×10^{-9} per site per yr, which, when converted into rate (v_{aa}) defined at the protein level, corresponds to $\sim 5.0 \times 10^{-9}$ per residue per yr (as the number of replacement sites per codon was estimated to be ~ 2.4 on average, $v_{aa} = 2.4 v_A$). This rate is high, almost comparable with that of immunoglobulin⁸. To estimate v'_A , two different speeds of evolution were assumed: one (v'_A) in the line from a_0 to G in Fig. 1; the other (v_A) in the line from a_0 to functional genes (X) other than the G gene. This gives the equation

$$v_A t_0 + v'_A t_0 = K_A^c(G-X) \quad (2)$$

where t_0 is the date of node a_0 and $K(G-X)$ is the sequence difference between G and X. Solving this for each functional gene and averaging the resulting values of v'_A , we obtain $v'_A = 0.17 \times 10^{-9}$ per site per yr or $v'_{aa} = 0.41 \times 10^{-9}$ per residue per yr, which is similar to the rate for insulin, a slowly evolving molecule⁸. It is remarkable that the IFN- α G gene, a direct descendant of the common ancestor of all the eight distinct classes, accumulates far fewer base substitutions at the replacement sites than other IFN- α genes, at a rate of about one-tenth that of the other genes, suggesting the presence of strong selective pressure depending on the unique function of the encoded protein.

The Hu IFN- α multigene family contains a pseudogene (IFN- α E) which shows close sequence homology to the IFN- α G gene, suggesting that it was derived from the G gene during evolution. This represents the first evidence of a pseudogene transcribed into mRNA³. Thus it is interesting to determine whether or not the IFN- α E gene shows strong sequence divergence similar to that of other pseudogenes²¹⁻²⁶. We have calculated differences between gene sequences from their joint

node (a_6 of Fig. 1). Designating 'O' for node a_6 and X for any one of IFN- α A, B, C, D, F and H genes, we have a set of equations: $K(X-G) = K(O-X) + K(O-G)$, $K(X-E) = K(O-X) + K(O-E)$ and $K(G-E) = K(O-G) + K(O-E)$, where $K(O-G)$, for example, represents the sequence difference between O and G. Solving this, we obtain $K(O-G) = [K(X-G) + K(G-E) - K(X-E)]/2$, $K(O-E) = [K(X-E) + K(G-E) - K(X-G)]/2$ and $K(O-X) = [K(X-G) + K(X-E) - K(G-E)]/2$. Using the K_S values in Table 1, we calculated the three differences for each X. The mean values ($\pm$ s.e.) of the three differences were $K_S(O-G) = 0.075(\pm 0.031)$, $K_S(O-E) = 0.126(\pm 0.040)$ and $K_S(O-X) = 0.149(\pm 0.044)$. Similarly, $K_A(O-G) = 0.011(\pm 0.006)$, $K_A(O-E) = 0.061(\pm 0.014)$ and $K_A(O-X) = 0.047(\pm 0.012)$ (the standard errors were calculated by assuming a Poisson distribution for the total number of base changes and by considering, on average, 308.6 replacement sites and 78.4 synonymous sites in the sequences compared). This result suggests that the E gene accumulated more substitutions than the functional genes at both the synonymous and replacement sites, although the standard errors seem rather large. In addition, the rate of change at the replacement sites of the pseudogene, relative to the standard genes, increases more than the rate of change at the synonymous sites: the mean values are $K_A(O-E)/K_A(O-G) (= 5.6) > K_A(O-E)/K_A(O-X) (= 2.9) > K_S(O-E)/K_S(O-G) (= 1.7) \sim K_S(O-E)/K_S(O-X) (= 1.9) > 1$, where $K(O-X)$ is $K(O-X)/2.25$, as, according to the phylogenetic tree of Fig. 1, the time interval between O and X is estimated to 2.25 times longer than that between O and G. These relationships are the same for each of the six different X genes. These patterns of base substitutions are similar to those of other pseudogenes analysed previously, including α -globin pseudogenes of man and mouse, β -globin pseudogenes of rabbit and mouse and immunoglobulin V_k pseudogene of man¹⁰, suggesting that pseudogenes show strong sequence divergence compared with functional genes, regardless of whether the mRNAs are transcribed.

A possible interpretation of these results is that in most functional genes, the replacement sites are more constrained than the synonymous sites. However, in pseudogenes the loss of functions frees the gene from several selective constraints, thereby allowing accumulation of substitutions almost uniformly over all the regions at rate higher than the rate of change at the synonymous sites of functional genes, the most rapidly evolving component (see refs in ref. 13). The fact that $K_S(O-E) > K_S(O-G)$ suggests that even synonymous sites of functional genes are constrained, although presumably only weakly¹⁰. The suggestion that the IFN- α E pseudogene has evolved faster than functional genes may support the neutral theory²⁷, its rate of

evolution may approximate to a limiting rate predicted by the neutral theory^{9-11,28}.

In human α - and β -globin, and rabbit and mouse β -globin gene clusters, pseudogenes are found between functionally distinct genes, that is, between the embryonic or fetal and adult genes^{23-25,29}. The suggestion that the IFN- α E pseudogene was derived from the IFN- α G gene is of interest in this regard. The fact that the v_A of the G gene is smaller than that of other genes suggests that the functions of the proteins encoded by them differ. Although the complete linkage arrangement of the Hu IFN- α multigene family has not been established, this suggests that the E pseudogene may be located between the G gene and the other groups of genes. Furthermore, IFN- α C, F' and H genes (group I) show close sequence homology, suggesting that they are closely linked. It would be interesting to determine whether further pseudogenes reside between this group and group II (A, B and D) or III (G). This would provide further insights into the mechanisms by which pseudogenes arise during evolution.

Goeddel *et al.*³ found size heterogeneity in the 3'-noncoding regions of the eight distinct Hu IFN- α mRNA sequences. To trace the evolutionary origin of such heterogeneity, we have compared the untranscribed 3'-flanking sequences of Hu IFN- α_1 (a member of class D)² with the IFN- α B, C, E, F and H mRNA sequences in the region corresponding to that immediately after the poly(A) sites of IFN- α A, D and G mRNAs, the shortest mRNAs. We found remarkable homology: the untranscribed 3'-flanking sequence of IFN- α_1 is 74% homologous to the longest B mRNA sequence in the corresponding region. In the IFN- α_1 sequence, the hexanucleotide AATAAA, which is thought to be involved in mRNA polyadenylation and processing³⁰, and its variant ATTAAA were found at positions 37-42 and 115-120, respectively (see Fig. 2). Presumably the IFN- α A and G genes also contain such hexanucleotides in this region. It seems likely that the presence of multiple hexanucleotides allowed the extension of the 3'-noncoding regions of the IFN- α genes during evolution, and thus gave rise to size heterogeneity of the mRNAs. Interestingly, two hexanucleotides precede the poly(A) sites in the B and F mRNAs, suggesting that the multiple hexanucleotides in each mRNA are recognized with varying efficiency, giving rise to mRNAs having 3'-noncoding regions of different sizes from a single gene³¹.

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H-2-like genes in the *Tla* region of mouse chromosome 17

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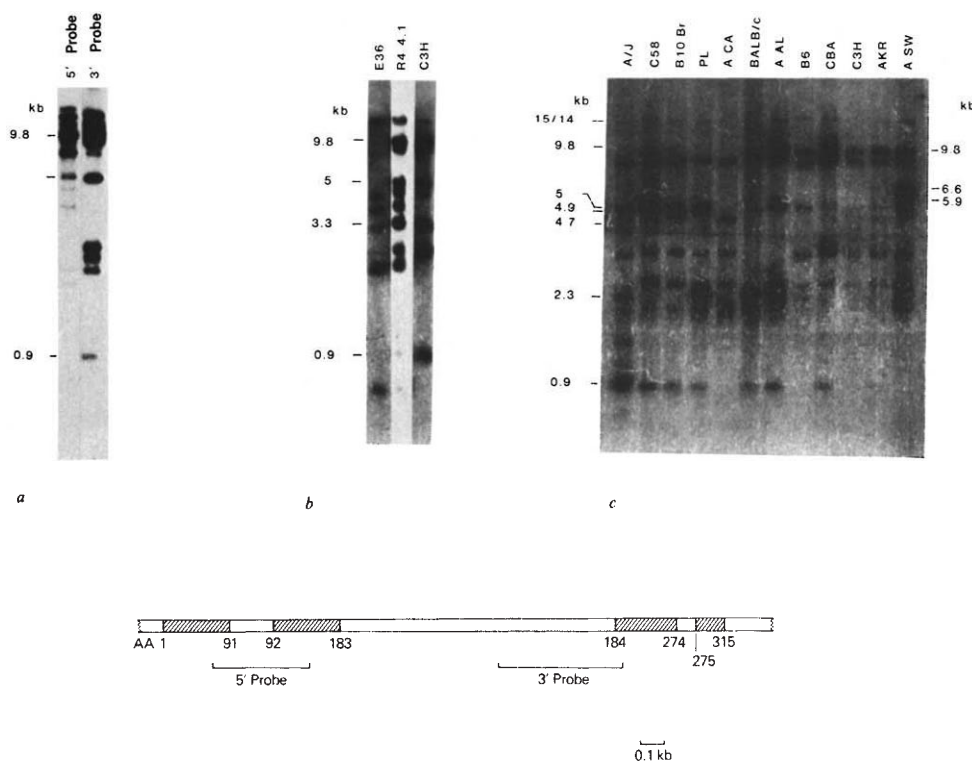
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H-2K, H-2D, H-2L, Qa-2 and TL are all members of a closely related family of cell-surface glycoproteins. The H-2 molecules (major histocompatibility antigens) are extremely polymorphic, expressed on almost all cell types and play an important part in graft rejection¹⁻⁴. In contrast, Qa-2 and TL are much less polymorphic, and have a limited tissue distribution^{4,5}. Nevertheless, all these molecules are similar in that they consist of two chains, one of which is the invariant β_2 -microglobulin (molecular weight (MW) 11,500), the other a 45,000-MW glycopolypeptide^{2,6-10}. The 45,000-MW H-2K and H-2D map within 0.3 centimorgans of each other in the major histocompatibility complex (MHC) on chromosome 17 (ref. 1). The Qa-2 and TL chains are encoded by genes in the closely linked *Tla* region. Recently, recombinant DNA techniques have been used to study the family of genes encoding H-2-like molecules¹¹⁻¹⁴. The finding that cloned H-2 cDNA hybridizes to 10-20 restriction fragments of genomic DNA has led to the conclusion that there are 10-20 H-2-like genes in the murine genome^{11,12}. Here we present evidence that most of these genes are encoded on chromosome 17 and that at least three H-2-like genes map outside the MHC in the *Tla* region. We also confirm and provide further evidence that there are 10 to 15 H-2-like genes in the mouse genome.

Initial studies of the number of genes in the H-2 family^{11,12} used cDNA probes complementary to portions of the H-2-like mRNA. Thus, the precise relationship between the structure of the probe and the sequences detected in Southern blotting experiments is uncertain. If these probes span intervening sequences that contain relevant restriction sites, they would be expected to hybridize to several genomic DNA fragments from the same gene, thus overestimating the number of genes. To avoid this we used probes from the cloned genomic DNA to identify *Bam*HI restriction fragments of BALB/cN myeloma DNA that encode H-2-like genes (Fig. 1a). The 3' probe (Fig. 1d) and a probe that encodes amino acids 194-283 (and intervening sequence) identify similar sets of restriction fragments (D.H.M. and J.G.S., unpublished observations). In contrast, the 5' (Fig. 1d) and 3' probes do not identify the same fragments of *Bam*HI-digested BALB/c myeloma DNA (Fig. 1a). Thus, the 5' and 3' portions of some H-2-like genes lie on different *Bam*HI restriction fragments. However, each probe identifies 10-15 fragments. This is a minimum estimate of the gene number as we cannot be certain that our probe detects all the H-2 genes in the hybridization conditions used and because some of the larger fragments contain two H-2-like genes (unpublished observation). These fragments may represent inactive or pseudogenes, and there may be other MHC transplantation genes that are not recognized by the probes. Kourilsky *et al.*¹¹ detected a similar pattern of *Bam*HI restriction fragments in BALB/c DNA using a probe ~85% homologous to the 3' probe used here.

The fact that the H-2L, H-2D and H-2K genes are all closely linked on chromosome 17 in the MHC does not mean that all H-2-like genes are necessarily encoded on this chromosome. To determine which H-2-like genes are encoded on chromosome 17, we have analysed the DNA of the cloned somatic cell hybrid, R4 4.1 (ref. 15), which contains a full complement of Chinese hamster chromosomes but only chromosome 17 derived from

Fig. 1 Southern blot analysis of genomic DNA probed with fragments derived from an *H-2*-like gene. The structure of the *H-2*-like genomic clone and the 5' and 3' probe fragments derived from it are shown in *d*. This clone was isolated from the MOPC 41 (BALB/c) Charon 4A library using a fragment of a human HLA genomic clone as probe (G.A.E. and J.G.S., in preparation). This cloned genomic DNA fragment encodes a protein that is 70–90% homologous to H-2K^d, H-2D^d and H-2K^b. The indicated *Sau*3A fragments were cloned in M13mp7 as described elsewhere¹⁸. The subcloned fragment was separated from the replicative form (double-stranded DNA) by digestion with *Eco*RI, and purified on disulphide-linked polyacrylamide gels¹⁹. DNA was prepared from: BALB/c myeloma (NP-2) DNA; E36 and R4 4.1 from cultured cells; and C58 from the R1.1 cultured cell line¹⁰. All other DNA was extracted from livers of mice obtained from either Dr I. Egorov (BALB/cBy) or Dr J. Berzofsky (B10.Br), Jackson Laboratories, or *Tla* and *Qa* recombinant strains^{4,5}. Digestion of the DNA *Bam*HI endonuclease, electrophoresis in 0.8% or 1.0% agarose gels (*a*), blotting to nitrocellulose filters, hybridization to the nick-translated probes (specific activity between 2.0 and 4.0×10^8 c.p.m. per μ g), washing in 0.015 M NaCl, 0.0015 M Na citrate at 50 °C, and autoradiography were all as described elsewhere^{21–23}. Sizes of fragments (in kilobases) are indicated. *a*, BALB/c DNA (NP-2) probed with 5' and 3' probes; *b*, Chinese hamster E36, mouse $\times$ hamster hybrid clone R4 4.1, and C3H DNAs probed with the 3' probe; *c*, genomic DNA from the indicated strains (BALB/c, BALB/cBy liver; B6, C57BL/6); *d*, structure of the *H-2*-like genomic clone and derivation of its probes. Shaded areas indicate coding blocks and adjacent amino acids are indicated.



the mouse L cell parent. The *Bam*HI restriction fragments identified in digests of genomic DNA from E36 (Chinese hamster cell), R4 4.1 and C3H (parent of the L cell) are indicated in Fig. 1*b*. Fragments derived from R4 4.1 DNA but not E36 DNA must be encoded on chromosome 17. There are many genes encoded in the hamster genome that hybridize to the *H-2* probe. However, the chromosomal location of mouse-derived restriction fragments that co-migrate with hamster-derived restriction fragments cannot be determined from this experiment. Nevertheless, there are at least seven murine-derived restriction fragments in the R4 4.1 DNA digests. Thus at least seven of the 10–15 *H-2*-like genes are encoded on murine chromosome 17.

A unique feature of the *H-2* antigens is their extreme polymorphism among inbred mice. Using the 3' probe to examine the *Bam*HI-derived restriction fragments from a series of inbred mouse strains (Fig. 1*c*), we found that these patterns are similar overall. The number of restriction fragments encoding *H-2*-like genes was similar in the different strains. Thus, the number of *H-2*-like genes does not vary widely among the inbred strains examined. In addition, many fragments (~50%) were common to different mouse strains both in the *Bam*HI digest shown here, and in digests using other enzymes (*Xba*I and *Eco*RI) (unpublished observations). However, comparison of the *H-2K* and *H-2D* types (Table 1) of these strains and their restriction fragment pattern (Fig. 1*c*) suggests that there are only a few fragments specific to a *H-2* haplotype. For example, A.SW has two unique fragments of 6.6 and 5.9 kilobases (kb) not found in other strains. These *H-2*-specific fragments may reflect differences in the major histocompatibility antigens of these mice.

There are differences in three restriction fragments between these mouse strains that have a distinct pattern, but which do not reflect the *H-2* type of a particular strain. For these cases, if a strain lacks a particular restriction fragment, it is replaced by another, compensating fragment. The surprising feature is that the replacement fragment is the same in several strains. For example, five strains have a 2.3-kb fragment (A/J, PL, BALB/c, A.AL, B6) while the strains that lack this fragment (C58, B10.Br, A.CA, CBA, C3H and AKR) have a 4.7-kb fragment.

A.SW is an exception to this rule, lacking both fragments. There are two further examples of this type of compensatory polymorphism: (1) nine strains (A/J, C58, B10.Br, PL, BALB/c, A.AL, CBA, C3H and AKR) have 0.9-kb and 14-kb fragments, while three strains (A.CA, B6, A.SW) replace these with a 15-kb fragment. (2) A 9.8-kb fragment is found in BALB/c, A.AL, B/6, CBA, C3H, AKR and A.SW and is replaced by a 4.9-kb fragment in A/J, C58, B10.Br and PL. A.CA appears to lack the compensatory 4.9-kb fragment. From these patterns we make the following conclusions: (1) Each of these fragments, and their compensatory fragments, represents a single mutational event that affects a single *H-2*-like gene or its flanking sequence. (2) Apparently, each band identified in the Southern

Table 1 Phenotypes of mouse strains

Strain	H-2K	H-2D	Qa-2,3	Tla
A	k	d	a	a
C58	k	k	b	a
B10.Br	k	k	b	a
PL	u	d	ND	a
A.CA	f	f	b	d
BALB/c	d	d	a*	c
A.AL	k	d	ND	c
B6	b	b	a	b
CBA	k	k	b	b
C3H	k	k	b	b
AKR	k	k	b	b
A.SW	s	s	a	b

The phenotypes for the inbred mouse strains are taken from Klein *et al.*¹⁷. ND, phenotype not determined.

*The phenotype of BALB/cJ is indicated here; the BALB/cBy (*Qa-2,3^b*) analysed in Fig. 1*c* apparently is an exception to the correlation of the presence of the 2,3-kb fragment with *Qa-2,3^a*. However, as it is likely that this is an independent mutation that occurred in a subline of BALB/c, one would not expect it to have the same restriction fragment pattern as other *Qa-2,3^b* strains. (Based on the genomic blot analysis, we can make tentative assignments of the *Qa-2,3* and *Tla* types of the unassigned strains as follows: PL, *Qa-2,3^a*; A.AL, *Qa-2,3^a*.)

blot corresponds to a single fragment in the mouse genome. Presumably, identical multiple mutations would not occur in different genes. (3) Each of these mutations must have occurred before the inbred lines were separated from each other. As several strains (for example, BALB/c, AKR and PL) have distinct origins, we suggest that these mutations occurred in the wild mouse population. The fact that these patterns do not correlate with the H-2 type of the strains suggests that either the mutations segregated independently of the H-2 type during evolution, or the H-2 haplotypes have been generated by independent mutation since the original mutations occurred.

To test the hypothesis that these polymorphisms segregate with other known genes encoded in chromosome 17, we compared the Qa-2,3 and Tla phenotypes of these strains (Table 1) with their restriction fragment patterns (Fig. 1c). The 4.7-kb fragment was found in the Qa-2^b strains while the 2.3-kb fragment was found in Qa-2^a. The 9.8-kb fragment was found in all Tla^b and Tla^c strains and the 4.9-kb in all Tla^a strains. Furthermore, A.CA, the only strain of the Tla^d haplotype examined, lacks the 9.8-kb as well as the 4.9-kb fragment. This suggests that the mutation separating Tla^b from Tla^c occurred more recently than the mutation distinguishing them from Tla^a. The presence of the 0.9-kb fragment does not correlate with the H-2, Qa-2,3 or Tla phenotypes.

The correlations suggested by examination of polymorphisms in inbred strains of mice might be misleading: the finding of a fragment in one inbred line and its replacement in another might reflect the history of these strains or the chance co-migration of several restriction fragments. Fortunately, several congenic strains of mice have been isolated which have undergone recombination in the vicinity of the Qa-2,3 and Tla genes^{3,9}. We have examined the DNAs of these strains to determine the map location of the restriction fragment polymorphisms (Figs 2, 3). Again, the 2.3-kb/4.7-kb polymorphism maps with the Qa-2,3 phenotype, and the 9.8-kb/4.9-kb fragments with the Tla phenotype. Recently, Steinmetz *et al.* have mapped a 2.7-kb *Bam*HI fragment to the Qa-2,3 region¹⁶. The 0.9-kb polymorphism does not segregate with either the Qa-2,3 or the Tla phenotype. However, these recombinant strains allow us to map this polymorphic H-2-like gene to the left of the Tla gene (as the fragment is found in A.Tla^b, but not in B6) but to the right of H-2D (as the fragment is found in B6.K1 but not in B6.Tla^a).

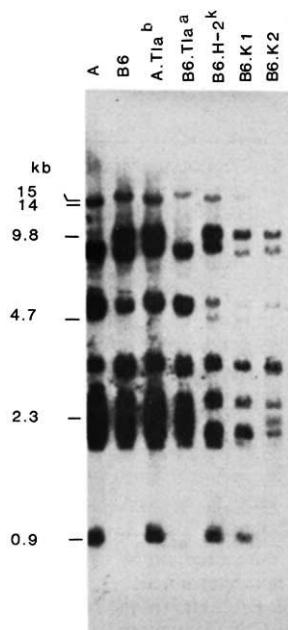


Fig. 2 Restriction fragment analysis of DNA from Tla and Qa region of congenic mouse strains. DNA from the indicated strains was analysed as described in Fig. 1 legend. Band intensity is a complex function of various factors. The greater intensity of the 2.5-kb fragment in strain A is a more usual result than the faint intensity of the same DNA seen in Fig. 1.

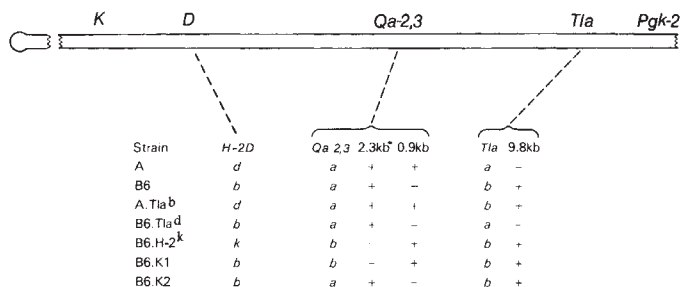


Fig. 3 Mapping of the Tla region polymorphism on chromosome 17. A schematic map of the relevant region on chromosome 17 is drawn to scale after Klein¹. The distance from K to D is 0.33 cm, and from D to Tla 1.3 cm. Qa-2,3 maps between D and Tla but its precise location is unknown. * Size of fragment indicated in Figs 1c and 2.

The presence of a 14-kb fragment that moves to 15 kb in the strains lacking the 0.9-kb fragment suggests that either this polymorphism contains a *Bam*HI site or that there are two H-2-like genes within 15 kb of each other. Thus, there are at least three H-2-like genes encoded in the Tla region, although these genes may not be Qa-2,3 and Tla.

These studies, using cloned genomic fragments from an H-2-like molecule as a molecular probe demonstrate that: (1) there are at least 10–15 H-2-like genes encoded in the mouse genome; (2) at least seven of these are encoded on chromosome 17; and (3) there are at least three H-2-like genes encoded outside the MHC which map to the Tla region. Future analysis of cloned H-2-like genes will permit a comparison of genes from the MHC with the Tla-encoded genes defined here. The basis for differences in the polymorphism and expression of the MHC and Tla-encoded genes may be reflected in their nucleotide sequences.

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Note added in proof: Subsequent studies have demonstrated that the molecular probes used in these experiments are derived from the H-2L^d gene²⁴.

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MATTERS ARISING

A pitfall in random sampling

MANY statistical tests depend on random samples. A random sample can be defined¹ as a sample from a distribution (for a univariate distribution this is an area) in which each point or neighbourhood has the same probability of being chosen. It is therefore necessary to pay close attention not merely to the process of sampling, but also to the nature of the distribution itself. It is sometimes easy to misinterpret a distribution and thereby invalidate the resulting test. For example, one can treat as stationary a distribution which may change over time. So stated the trap is obvious, but it can be subtle and occasionally catches excellent biologists. The question is totally different from estimating the shape or position of an average distribution determined *post facto* from a non-stationary process. Such an average distribution exists statistically but may have no causal meaning; an obvious example is the joint distribution of annual income of all residents of London since 1800.

Cole² criticizes the work of Evans and Murdoch³, who showed that herbivorous insect species in a community are a roughly constant proportion of all insect species over a season, despite much turnover. They therefore showed that the distribution is stationary, an important result. Cole not surprisingly found that Evans and Murdoch's data are not significantly different from what they would be if they were random samples of the same size from the total species pool. He then concluded that the data are irrelevant to ecological processes.

The fallacy is in assuming that there is a uniform pre-existing population from which samples are drawn (commonly without replacement, which affects the statistics in a subsidiary way). However, the very existence of such a stationary population is the interesting question, because it implicitly assumes that the relevant properties (here, diet) of the species sampled at different times are the same. Otherwise there would be no overall distribution which could be sampled randomly, and no reason to expect approximate constancy over time in the proportion of herbivores.

Thus, the results of Evans and Murdoch³ do add support for the idea of a dynamically maintained balance in trophic structure. This balance also has other aspects, notably in energy flow, and its control remains a central problem in ecology⁴.

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Anomalous ¹³C depletion in Precambrian organic carbon

SOME isotopic analyses of Precambrian reduced carbon which is strongly depleted in the heavier ¹³C isotope have been reported by Schoell and Wellmer¹. They have argued that these anomalous graphitic carbons might be isotopically light as the result of a fractionation related to methane bacterial action in the Precambrian. This argument is quite reasonable insofar as it assumes that the organic carbon in most Precambrian and early Phanerozoic sediments is itself normal. However, the average isotopic value of such reduced carbon is normal only from the experimental standpoint. When placed in context with the algal biota presumed to have been responsible for its origin and overall composition, it is also anomalously depleted of ¹³C.

In Fig. 1 of ref. 1, and in other similar compilations^{2–5}, the mean values for $\delta^{13}\text{C}(\text{PDB})$ cluster around –24 to –28‰. Such values appear quite normal when compared with the reduced carbon of modern C₃ terrestrial plants^{3,4,6,7}. However, a substantial land plant community did not develop until the Silurian, so that when compared with the reduced carbon from modern marine algae and algal mats, these 'normal' Precambrian isotopic ratios become disturbingly anomalous, and are especially so for the carbonate stromatolites^{5,8}. Present-day marine algal organic carbon is significantly heavier isotopically, with mean values ranging from –18 to –20‰ (refs 3, 4). Modern marine algal mats are even heavier^{8–12}, with values ranging from –8 to –19‰. This difference between the average terrestrial isotopic values and the average marine values of reduced carbon is so striking that it has been used to evaluate the impact of land-derived organic matter in modern marine sediments^{13,14}.

In the absence of a significant terrestrial biota in the Precambrian, how can average values ranging from –24 to –28‰ be explained? Those who have discussed this problem (see refs 3, 8, 10, 12, 15) have variously concluded that the depletion might be the results of: preferential diagenetic enrichment of isotopically light (lipid) fractions; a higher CO₂ partial pressure in the Precambrian environment; or perhaps different primitive metabolic fractionations.

When compared with data on ancient organic matter having both carbon isotopic and H/C atomic ratios^{2,16,17}, recent

laboratory-simulated thermal maturation experiments on algal mat kerogen¹¹ do not support the diagenetic argument. In these experiments only the most immature algal mat kerogens showed any tendency towards ¹³C depletion; all mature kerogens (H/C atomic ratios <1.0) were enriched in ¹³C.

Some laboratory experiments with marine algae grown under elevated CO₂ (1.5 to 3.5%) have produced carbon isotope ratios consistent with the idea of an increased level of CO₂ in the Precambrian^{10,15}. But the low cell densities involved and the absence of a potential for bicarbonate-derived CO₂ in these experiments makes them doubtfully applicable to the conditions in which the carbonate stromatolite mats of the Precambrian thrived.

While the very depleted values reported by Schoell and Wellmer¹ and others² are certainly anomalous in their own right, and may be the result of Precambrian biogenic methane, a more important anomaly remains. Until the average isotopic composition of organic carbon in a variety of Precambrian and Phanerozoic marine deposits can be explained in a consistent fashion, interpretations of the data to infer the physiology of the organisms involved, or the environments in which they were living, must be considered as tentative. This is especially important to timing the onset of oxygenic photosynthesis in the early history of life¹⁸.

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SCHOELL AND WELLMER REPLY—Towe has raised some often repeated questions which, however, are not directly relevant to our paper. We aimed to explain why some Precambrian graphites are depleted in ^{13}C compared with the bulk of all other Precambrian graphites. We offered an explanation by suggesting local phenomena such as CO_2 from oxidized methane as a food source for the original biota, rather than worldwide phenomena or completely different geochemical processes. However, Towe's comment indirectly bears on our argument and deserves a brief reply.

Two questions have been raised: why some organisms, specifically marine algae, are enriched in ^{13}C compared with common kerogens; and why Precambrian and early Phanerozoic kerogens are isotopically more enriched in ^{13}C as compared with modern marine algae and algal mats.

These problems have already been discussed by previous workers^{1,2}, who concluded in part that marine algae as found today could not have been the predominant precursor of Precambrian reduced organic matter. Recent work on the Precambrian argues for an establishment of a flourishing yet primitive life already 3,500 Myr ago^{3,4}. Detailed work on marine phytoplankton⁵ revealed a range of carbon isotope fractionation between HCO_3^- and organisms ranging from -22 to -35% (the mean of which interestingly is -27.5%). The carbon isotopic composition was found to depend on the species and on local conditions (pool size effect). The above figures easily explain the range of 'normal' Precambrian reduced organic matter.

The observation of relatively heavy carbon in less than half a dozen modern algal mats hitherto investigated does not justify the general statement of Precambrian organic matter as being 'disturbingly anomalous'. On the contrary, we emphasize the isotopic similarity of Precambrian, Phanerozoic and recent organic matter and consider this similarity as an argument for the early establishment of biological carbon fixation based on the presently operating enzymatic pathways, that is as proof for (1) the extreme antiquity of CO_2 fixation (notably by the RuBP carboxylase reaction) and (2) the constancy of the isotopic composition of the Earth's pool of inorganic carbon (atmospheric CO_2 and marine bicarbonate).

We thank M. Schidlowski for careful reading of this manuscript.

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Are monozygotic twins similar due to genetic factors alone?

THE number of errors and inconsistencies in the paper by Gärtner *et al.*¹ raises doubts about the validity of their conclusions. In Table 1, the mean and standard deviation of 51-day-old monozygotic (MZ) males are incorrect and differ from the mean and standard deviation for the same data given in Table 2. For 51-day-old dizygotic (DZ) males the authors estimate the between-pairs component of variance as $+1.18$ when the correct value is either zero, since it is not significant, or negative. This error is repeated for the same data in Table 2 where in addition, the same error is made for body weight of DZ males, aged 61 days. Also in Table 2, the F ratios between estimates of the variance components s_b^2 for MZ and DZ twins are incorrect. The ratios are not F tests; the degrees of freedom attributed to them are incorrect as are, therefore, the probabilities. Furthermore, for reasons which are not discussed in the text and which are not obvious, these probabilities appear to be one-tail. Even if the between-pairs items had been compared using correct tests, it is difficult to see what conclusions could have been drawn for the many characters for which, according to Table 2, MS_w s differ significantly between MZs and DZs.

Incidentally, the estimates of MS_w cannot take the extremely small values that are given in Table 2 unless their true values are zero and the values given are rounding-off errors or arbitrary. The assumption of "random effects" is superfluous for the analysis used, and an explanation is required for pooling males and females for some characters and omitting females for others when the authors say that all statistics were calculated separately for males and females.

Finally, there are features of the data which are difficult to reconcile with the explanation given by the authors. For example, examination of body weight in females from 31 to 61 days shows that the DZs, as expected, retain their initial level of variability as reflected in the total variance. Indeed, this variance increases slightly with age. In complete contrast, on the same criterion the MZs are as variable as the DZs at day 31, but lose their variability as they age. Indeed at day 61, they display almost no variability at all. Since the main conclusion of this paper is that there is a lower level of variation within

the MZs than the DZs and that this can be attributed to events that occur early in life, it is difficult to see why these MZ females reached their maximum variability early in life and their minimum as adults.

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1. Gärtner, K. & Baunack, E. *Nature* **292**, 646–647 (1981).

GÄRTNER AND BAUNACK REPLY—Our results¹ show that pairs of sexually concordant isogeneic DZ twins differ more than pairs of MZ twins because of a non-genetic mechanism which modifies each zygote differently before the third cell division. We thank Jinks and his co-workers for drawing attention to a few errors in our paper. However, these errors do not affect our conclusion as the similarity within the pairs of MZ is clearly much greater than that within DZ twins of inbred mice whatever the method of statistical analysis. Therefore the valid criticism of Jinks *et al.* of the F values for testing the between variance components (s_b^2) does not influence our main conclusion. Nor do the two regrettable errors they mentioned in printing different numbers in Tables 1 and 2 for the 51-day MZ body weights and the absence of the minus sign for the DZ 51 and DZ 61 variance components.

Further discussion of the statistical procedure helps only to a very small degree. To shorten this discussion we are prepared to send to any interested scientist our original data for statistical analysis by a method of his/her own choice.

We do not accept the other criticisms. The first three variables of Table 2 (appearance of the hair, eye opening, ear opening) are data measured in whole days. All the MZ pairs exhibited the event on the same day. It seems unrealistic to compare the alteration of the variance in the MZ female twins as Jinks *et al.* suggest, because of the very small numbers of female MZ twins.

Further confirmation can only come from the results of similar experiments in other laboratories. However, further experiments in our own laboratory using twins produced since our original paper, together with many additional characters, support our conclusions.

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BOOK REVIEWS

Once a jackass...

Marie Jahoda

IN July 1974, James Watson of *Double Helix* fame was one of the 11 eminent scientists who asked publicly for a voluntary moratorium on two types of experiment involving genetic manipulation. In 1979 Watson described the signatories as "jackasses" who displayed a complete lack of scientific judgement in asking for the moratorium. What happened in between, before and after these dates forms an extraordinary episode in the history of science. Confirming all one's prejudices about national character, events unfolded as a mixture of drama, confrontations, public soul-searching and farce, with the participation of a large and excited lay public in the United States; the issues were the same in England, but working parties rather than public meetings wrestled with them and lay participation was limited to a few

The DNA Story: A Documentary History of Gene Cloning. By James D. Watson and John Tooze. Pp.605. ISBN 0-7167-1292-X. (W.H. Freeman: 1981.) \$24.95, £17.30.

individuals on GMAG, the Genetic Manipulation Advisory Group (of which I was one). Lest this contrast between the phlegmatic English and the excitable Americans foster unwarranted interpretations, it should be said immediately that it was the American response that crystallized the questions and dilemmas that the marvellous advances in genetic manipulation present.

In this beautifully produced and illustrated volume consisting largely of facsimile documents, Watson and Tooze leave it to the reader to infer the basic questions but

emerge with only one clear answer: leave genetic manipulation to the scientists; no special foreseeable hazards are involved, hence no special regulations are required. Apparently the vast majority of the biological establishment in the United States now agrees; but not all, and some Nobel Prize winners are among the dissenters. That scientists disagree among themselves and change their minds is not news. Watson's recantation confirms that even great scientists are only human. Robert Sinsheimer, however, changed his mind in the opposite direction. Everybody's right to be a "jackass" once in a while admitted, how can one decide who is one when? Only the future will tell, but if the Sinsheimers are right, the answer may come too late.

In less colourful terms than Watson uses in his pungent and engagingly informal prose, the question is whether those at the very forefront of science can foresee the consequences of their achievements. The answer is an unqualified no. One remembers Rutherford on splitting the atom. Even Watson himself, talking about the double helix, says "... we did not foresee any immediate practical consequences for the world about us ..." (p.vii), yet his and Crick's discovery was the *sine qua non* of genetic manipulation. When the consequences became clear shortly after, the scientists themselves stopped in their tracks. Why? At the risk of being laughed out of court by these superior rational minds, I suggest that they were overcome by a metaphysical awe at their own power to fiddle with the very building blocks of life. Watson's self-derogatory explanation that he acted originally in a fit of liberal conscience is a bit superficial — conscience in scientists and others had better come not just in occasional fits. That metaphysical awe was akin to Oppenheimer's experience in witnessing the first atomic bomb explosion, and when it communicated itself to the public all hell broke loose. The unforgettable Mayor Velucci of Cambridge, Massachusetts, asked the Academy of Sciences to investigate whether the reported sighting of a monster could be due to genetic manipulation. Not the monster but the "sighting" revealed one theme underlying the debate that preoccupied not only Velucci but also some scientists and many others: the fear of the power of science that could deliberately, not accidentally, change the very nature of living creatures. The other theme, inherent in the position of Watson and his friends, concerned technicalities, not metaphysics, the avoidance of

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DEPARTMENT OF MOLECULAR BIOPHYSICS
AND BIOCHEMISTRY

July 17, 1973

Dr. Philip Handler, President
National Academy of Sciences
2101 Constitution Avenue
Washington, DC 20418

Dear Doctor Handler:

We are writing to you, on behalf of a number of scientists, to communicate a matter of deep concern. Several of the scientific reports presented at this year's Gordon Research Conference on Nucleic Acids (June 11-15, 1973, New Hampton, New Hampshire) indicated that we presently have the technical ability to join together, covalently, DNA molecules from diverse sources. Scientific developments over the past two years make it both reasonable and convenient to generate overlapping sequence homologies at the termini of different DNA molecules. The sequence homologies can then be used to combine the molecules by Watson-Crick hydrogen bonding. Application of existing methods permits subsequent covalent linkage of such molecules. This technique could be used, for example, to combine DNA from animal viruses with bacterial DNA, or DNAs of different viral origin might be so joined. In this way new kinds of hybrid plasmids or viruses, with biological activity of unpredictable nature, may eventually be created. These experiments offer exciting and interesting potential both for advancing knowledge of fundamental biological processes and for alleviation of human health problems.

Certain such hybrid molecules may prove hazardous to laboratory workers and to the public. Although no hazard has yet been established, prudence suggests that the potential hazard be seriously considered.

A majority of those attending the Conference voted to communicate their concern in this matter to you and to the President of the Institute of Medicine (to whom this letter is also being sent). The conferees suggested that the Academies establish a study committee to consider this problem and to recommend specific actions or guidelines should that seem appropriate. Related problems such as the risks involved in current large-scale preparation of animal viruses might also be considered.

A list of participants in the Conference is attached for your interest.

Sincerely yours,

Maxine Singer and Dieter Söll

The debate widens: the letter expressing concern over aspects of recombinant DNA research written by Maxine Singer of NIH and Dieter Söll of Yale to Philip Handler, President of the National Academy of Sciences. This letter, and another to *Science*, were the upshot of the 1973 Gordon Conference on Nucleic Acids, a private meeting attended by some 130 molecular biologists.

accidents, not the unforeseeable outcome of their deliberate efforts. That the technical theme won in the end is due not only to the greater sophistication of its proponents but also to the fact that the meta-physical question is unanswerable — which does not, however, eliminate it. Fears and hopes were vastly exaggerated and opinions about the unforeseeable polarized. Scientists on both sides of the public debate spent for years large proportions of their time not in research but in lobbying Congress and state governments, and in writing and speaking for the public media. Unused though they were to political manoeuvrings, those on Watson's side did pretty well. The NIH guidelines were significantly relaxed, as were GMAG's procedures, and the scientists, bruised but relieved, could return to their work. The issues raised by the episode remain, however.

During these eventful years some suggested that all genetic manipulation should be stopped. Even if it were desirable, this cannot be done. Short of nuclear catastrophe the acquisition of scientific knowledge is an irreversible process; for better or worse we have to live with it. The question is how? Watson is against all regulations, as is Norton Zinder who believes that recombinant DNA research is "... as safe as most other biological research and safer than that dealing with a number of very dangerous micro-organisms ..." (p.201). These scientists defend their wish to be left alone by pointing to other uncontrolled hazards and regard the good sense of the scientific community as sufficient guarantee of safety. Such arguments are suspect because they are so clearly interwoven with self-interest. Richard Novick expressed this best when he declared himself unable to distinguish between the following two statements as bases for his belief that the experiments were safe:

1. I am convinced they are not dangerous, and therefore it is ok to do them; or 2. I have convinced myself they are safe precisely because I want to do them! [p.149].

If the whole episode demonstrates one thing it is that there is no harmonious scientific community interested only in the advancement of knowledge and its benefits for mankind. Yes, there is passionate commitment to driving forward their breath-taking discoveries; but there is also ambition, jealousy, lack of foresight, moral ambiguity and arrogance in these scientists, and there is absolutely no reason to assume that they are any better in these matters than we of a lesser breed. It seems reasonable to require that some people whose self-interest is not vested in genetic manipulation should be involved in monitoring these experiments.

This, of course, is easier said than done. Lay participation, as in the United States at the height of the controversy, may only have strengthened the irrational and anti-science trends in society. It has not bridged

the enormous gap in knowledge between the scientists and the general public. Lay participation as in England is token participation, symbolic rather than real. What can a lay person do, when scientists disagree about the containment category of a given experiment, but side with the one with the bluer eyes? Perhaps such frustration about one's technical incompetence will be mitigated when issues arise about which we all are equally competent or incompetent. One such issue, though not restricted to genetic manipulation, arises over the possibility of cloning human beings. Sir Peter Medawar (p.246) considers this well nigh impossible but reports that Jacques Monod did not. John Maynard Smith agrees with Monod.

Irish rocks reviewed

W.S. McKerrow

A Geology of Ireland. Edited by C.H. Holland. Pp.335. Hbk ISBN UK 0-7073-0269-2, ISBN US 0-470-27247-3; pbk ISBN 0-7073-0308-7. (Scottish Academic Press/Halsted: 1981.) Hbk £27.50, \$49.95; pbk £15.

MOST geologists know that the British Isles contain rocks of unusually diverse types and ages for such a small part of the world. Ireland is as varied and complex as any other part of these islands; thus it is good to be provided with a concise review of Irish geology. In the final chapter (on the history of Irish geology) we are reminded of the lean years between 1914 and 1947 when significant advances were made in only two branches of geology in Ireland: Tertiary igneous petrology, and Pleistocene stratigraphy and geomorphology. This was followed by a revival in which both academic and economic geologists, mainly from outside Ireland, played a part. In the past two decades Irish geologists (living in Ireland, if not all of Irish descent) have performed an increasing amount of the geological research in Ireland. The ten contributors to the book have each spent much or all of their professional careers in Ireland, eight in Dublin and two in Belfast, and each of them is an authority in his own field.

In the preface, Professor Holland points out that a lot has happened in our understanding of Irish geology since Charlesworth's *Historical Geology of Ireland* was published by Oliver & Boyd in 1963, but this book follows a very different pattern. While Charlesworth attempted to refer to every paper (good, bad and indifferent) on Irish geology, and thus is still a good source for the older literature, Holland has been much more selective; and while Charlesworth covered the whole country single-handed, Holland has gathered a team for the task. The authors each possess an individual style and range of expertise,

The great merit of Watson and Tooze's book is that it displays the arguments on all sides. It offers only personal, not general solutions to fundamental questions. The worst sequel to this whole exciting episode would be if young scientists decided to withdraw into their ivory towers; the best if it were regarded as a rehearsal for things to come, from which to learn how ill-prepared scientists and the public still are to face the issues that confront them jointly and how to improve their relations while there is time. □

Marie Jahoda is Professor Emeritus of Social Psychology and Consultant to the Science Policy Research Unit, University of Sussex. For two years she was a member of GMAG, representing the public interest.

but the editor has clearly stamped his authority on the book. Each chapter is a very readable, well-illustrated, resumé of our knowledge and understanding of Irish geology today. (Or is it yesterday? Most chapters make little reference to work later than 1976, and most fail to include references later than 1978).

In addition to the chapters on stratigraphy, there are sections on tectonics, igneous activity and geophysics, which might be expected in such a general review. More unexpected, especially to those geologists who regard drift as a nuisance, are the fascinating chapters on the Quaternary, which extend well past the 5,000 BP date of the first Irishmen, and are almost continuous, with no more than small non-sequences, through to the final, historical chapter.

Economic geology has played, and is continuing to play, a major part in Irish life, from the Bronze Age copper mines of County Cork to the search for offshore oil today. The review includes clear descriptions of the major metal mines, together with a summary of opinions on the possible origins of the ore bodies. The section on oil and natural gas contains a useful summary of the offshore geology around Ireland, from the Celtic Sea to the Rockall Plateau.

A Geology of Ireland will prove to be a useful book for the student who wishes to obtain a general view of Irish geology; it should therefore find a place in every geology library. But the research geologist, academic or economic, may not find all that he requires in the text, or, more seriously, in the references — the editor has limited them to a level which I would consider to be lower than satisfactory. The Geological Society of London *Special Reports* on the correlation of rocks in the British Isles do provide many of the missing references, but some of these are now even more out of date than the references listed in the book. □

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Joseph Henry: letters from America

Colin Russell

The Papers of Joseph Henry, Vol. 4: January 1838–December 1840, The Princeton Years. Edited by Nathan Reingold. Pp.475. ISBN 0-87474-792-9. (Smithsonian Institution Press, Washington DC: 1981.) \$30.

JOSEPH Henry (1797–1878) was an American physicist and a researcher in electricity and magnetism. He had the misfortune to be a contemporary of Faraday by whom he has been considerably overshadowed; even when he emerges from the penumbra of the great Englishman he is not seen as a major luminary in science, and one may wonder at the colossal expenditure of labour in this reprinting of his papers. If it may not be justified in terms of Henry's inherent scientific stature, the rationale would appear to lie in his position within the community of early nineteenth-century science, particularly in America. He knew almost everyone of importance on both sides of the Atlantic, and such people flit effortlessly in and out of his correspondence. To read his papers is to gain a richer understanding of the social milieu in which Henry lived and much of modern physics took shape.

During most of the period covered by this book (1838–1840) Henry's life was fairly uneventful, concerned mainly with his family at home or seriously fulfilling his calling as Professor of Natural Philosophy at Princeton. For this reason the present volume lacks something of the punch and zest of its predecessor (for review see *Nature* 286, 311; 1980), which dealt mainly with his grand tour of Europe. However there are compensations, and a large proportion of the book is concerned with his "records of experiments" from manuscript material. Some of his work was over-hasty as he wished to establish priority over Faraday, but here is an unself-conscious record of one man working under great pressure to unravel the complex phenomena of electromagnetism. Amongst his most significant discoveries of this time was that of electric induction across great distances, leading him to take the side of those who advocated an all-pervasive ether, thus anticipating in certain respects the more comprehensive theories of Maxwell.

In the correspondence a note that constantly reappears is that American science must at all costs be protected from the quacks and the charlatans (amongst whom he numbers the egregious Samuel Morse). In this respect New York was worse than Philadelphia, but the danger was endemic in a small beleaguered community of science that lacked the checks and balances of its older European counterparts. Much could be learned from Europe, of course, and America had its English-sounding Society for the

Promotion of Useful Arts, while the American Association for the Advancement of Science was consciously modelled on the British equivalent. But Henry (who had been unfairly savaged at a BAAS meeting a few months earlier) very much preferred a meritocracy of science to the arrangements in Britain, in which "wisdom" and "ignorance" were mixed and where the Society was "quite as aristocratical as the government of the nation". Thus we are offered a view of British science which is rather different from that purveyed by the popularizers and promoters in Britain itself.

As in previous volumes of this series there is a short introduction, very full footnotes (which include indications of variant readings) and an excellent index. Diagrams are reproduced in facsimile and there are several pages of portraits and illustrations. One or two editorial statements raise an eyebrow here and there (for example that the preparation of magnesium was "first reported" in 1831) and on p.310 two lines have been transposed. However, as we have come to expect, the standard of production is generally superb, with the luxury of footnotes at the bottom of the page or at the end of each document. When several publishers are charging twice as much for shorter works, with much less lavish presentation, this book is a rare bargain for all concerned with history of nineteenth-century physical science. □

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Standard methods in geomorphology

C. Kidson

Geomorphological Techniques. Edited by Andrew Goudie. Pp.416. Hbk ISBN 0-004-551022-3; pbk ISBN 0-004-551043-1. (George Allen & Unwin: 1981.) Hbk £25, \$60; pbk £12.95, \$29.95.

GEOMORPHOLOGY, the science which seeks to understand the processes which shape the surface of the Earth, lies in the borderland between geography and geology. In the United States it is firmly based in geology. In Europe, however, most of the recent major developments in the subject have, by and large, been the work of physical geographers. The British Geomorphological Research Group, for

whom this volume is edited, have, as a subject group of the Institute of British Geographers, been in the forefront of research in this field. Thus it is entirely appropriate that an attempt to bring together those techniques, both field and laboratory, most appropriate to the discipline should emanate from this active and distinguished group of geographers. In a sense, the book is a natural development from their successful series of *Technical Bulletins*, two-thirds of which are referred to in the text. It does, however, go well beyond the scope of that series.

A prime objective of a "manual of useful techniques" should be to provide a work of reference for those who wish to improve their technical repertoire. Kummel and Raup's *Handbook of Palaeontological Techniques* (W.H. Freeman, 1965) is an example of what can be achieved. In its better sections, *Geomorphological Techniques* matches the high standards set in that volume — Parts 3 (*Material Properties*) and 4 (*Process*) are as useful and authoritative summaries as the readers of the book could hope to find. In others, however, notably the first two parts on form and time, the techniques are enclosed in a matrix of theory and philosophy which suggest a textbook rather than a manual. In falling between two stools the value of these parts is reduced.

Inevitably, in a volume arranged on the basis of form, materials, process and time, and written by more than 30 authors, there are some techniques which have been inadequately dealt with or even omitted. Topographic survey methods such as levelling are superficially treated. Plane tabling, which one would have thought to be basic to students of landforms, is totally omitted. The collection of samples with the aid of augers, corers and drills is scarcely mentioned, with the notable exception of the section on glacial processes and that on peats and lake sediments. In addition, the coverage of tracer techniques is woefully inadequate.

Surprisingly, for a work of this calibre, there are some remarkably superficial statements. On p.8, for example, it is asserted that "During the predominance of denudation chronology, data acquisition was dominated largely by qualitative field observations and subjective map analysis". No one who had been in the field with Wooldridge or Cotton, or who had attended a class in cartographic analysis with Hollingsworth, could conceivably have made such a meaningless generalization.

Despite its imperfections this is a useful contribution to this vigorously growing science. It is the best available comprehensive treatment of the subject and is destined to be the standard work for some time to come. □

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Amino acids in clinical studies

A. Darbre

Amino Acid Analysis. Edited by J.M. Rattenbury. Pp.380. ISBN UK 0-85312-194-X; ISBN US 0-470-27141-8. (Ellis Horwood/Halsted: 1981.) £27.50, \$89.95.

WITH the appearance of the automated ion exchange column method of Spackman, Stein and Moore in 1958, amino acid analysis became one of the most important analytical techniques in biochemistry. Since that date there has been much research effort put into obtaining both faster and more sensitive analyses, and this has enabled the study of proteins, peptides and amino acids to be extended into many fields.

Proteins having unusual properties (e.g. anti-freeze protein) or specialized functions (e.g. receptor proteins on cell membranes) are being continually discovered and isolated, as are peptides and non-protein amino acids with important physiological and pharmacological properties.

This volume consists mainly of the papers given at a symposium, "Amino Acid Analysis in Clinical Chemistry and Medical Research", held in Edinburgh in June 1979. Thus the short title of the book is misleading — this is not a book for the reader whose primary interest is in protein

analysis or in methodology. The volume consists of 24 chapters and is divided into four parts. The first, on technical developments, contains 9 chapters which deal with analysis *per se* and review briefly the history of amino acid analysis, fluorimetric detection techniques, gas chromatographic methods, mass spectrometry, derivative spectroscopy and chemiluminescent nitrogen analysis. (The brief contribution on phenylthiohydantoin amino acids, however, does not have a logical place in this book.) The reader who may have accepted at face value widely disseminated statements that "today, it is possible to analyse picomole levels of amino acids in protein hydrolysates in 1h with the automatic loading of up to 80 samples" (p.138), with complete computation and print out of the results to an accuracy of 3 per cent (see p.132), should read the chapters on standards and accuracy (R. P. Ambler) and collaborative trials (A. P. Williams). These should induce in the researcher a much more critical approach to the "print-out" of results obtained from the computing integrator!

The *raison d'être* for this volume lies in the last 15 chapters, which have been divided into separate parts under the

headings of amino acid analysis in, respectively, physiological processes, systemic disease and congenital disorders. There is much of interest here, presented in easily readable form, but the emphasis is on results obtained in a physiological and clinical context, and not on the methods used. Thus there are chapters related to protein digestion and absorption (our knowledge here is still very inadequate), the effect of protein quality on plasma amino acid levels in low birthweight babies and plasma amino acids following parenteral nutrition of the newborn, and in chronic renal failure, acute liver failure and alcoholism. The specific analysis of 3-methylhistidine and hydroxyproline as a measure of protein breakdown, and amino acids as neurotransmitters are also discussed. Two contributions give generalized accounts of amino acid analysis in the study of inborn errors of metabolism, whilst others are devoted to homocystinuria, Wilson's disease and spina bifida.

All chapters include adequate references for further reading. But it should be noted that a new word — "exterioisable" — (p.153) has crept into the English language: this inborn error of grammar should be quickly eliminated. □

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Synmetals: a new branch to polymerization

Herman Mark

Semiconducting Polymers. By Marian Kryszewski. Pp.710. ISBN 83-01-00818-0. (Polish Scientific Publishers, Warsaw: 1981.) \$35.

FOR many years synthetic organic polymers have been of considerable interest and value for their mechanical and thermal properties such as rigidity, strength, elasticity, high softening range and chemical stability. With these properties they have been very useful in a variety of industries, textiles, films, sheets and plastics, for example. Several years ago, however, these materials underwent a surprisingly rapid development as "synmetals", that is, systems which can act as conductors or semiconductors. Their preparation and tailoring for the second application has now grown into a science and technology of considerable proportions.

Marian Kryszewski's *Semiconducting Polymers* is the first comprehensive treatment of this new field. It serves a two-fold purpose: first, it familiarizes the reader briefly with the basic principles of semiconduction, and then it describes how this intriguing property can be obtained with organic polymers.

There are two principles which serve to establish semiconductivity in such systems: the presence of polyconjugated chains and the availability of charge transfer complexes. The largest part of the book is reasonably devoted to the description of polyacetylene because this has been more closely investigated and has already led to semiconductors of wide potential application — f.i. in the construction of new types of photovoltaic cells, pace-makers and thin electrical batteries. Also included in the account of their preparation and behaviour are descriptions of their photoconductivity, magnetic characteristics and catalytic activity.

The author has been through his topic with great assiduity and diligence. For the generally interested reader he has provided a lucid and attractive story; the expert, too, will be grateful to him for the 3,000 or so references which constitute a comprehensive bibliography.

This is a remarkable book and, as the new field grows and unfolds, it may well one day be seen as a classic. □

Herman Mark is Dean Emeritus of the Polytechnic Institute of New York.